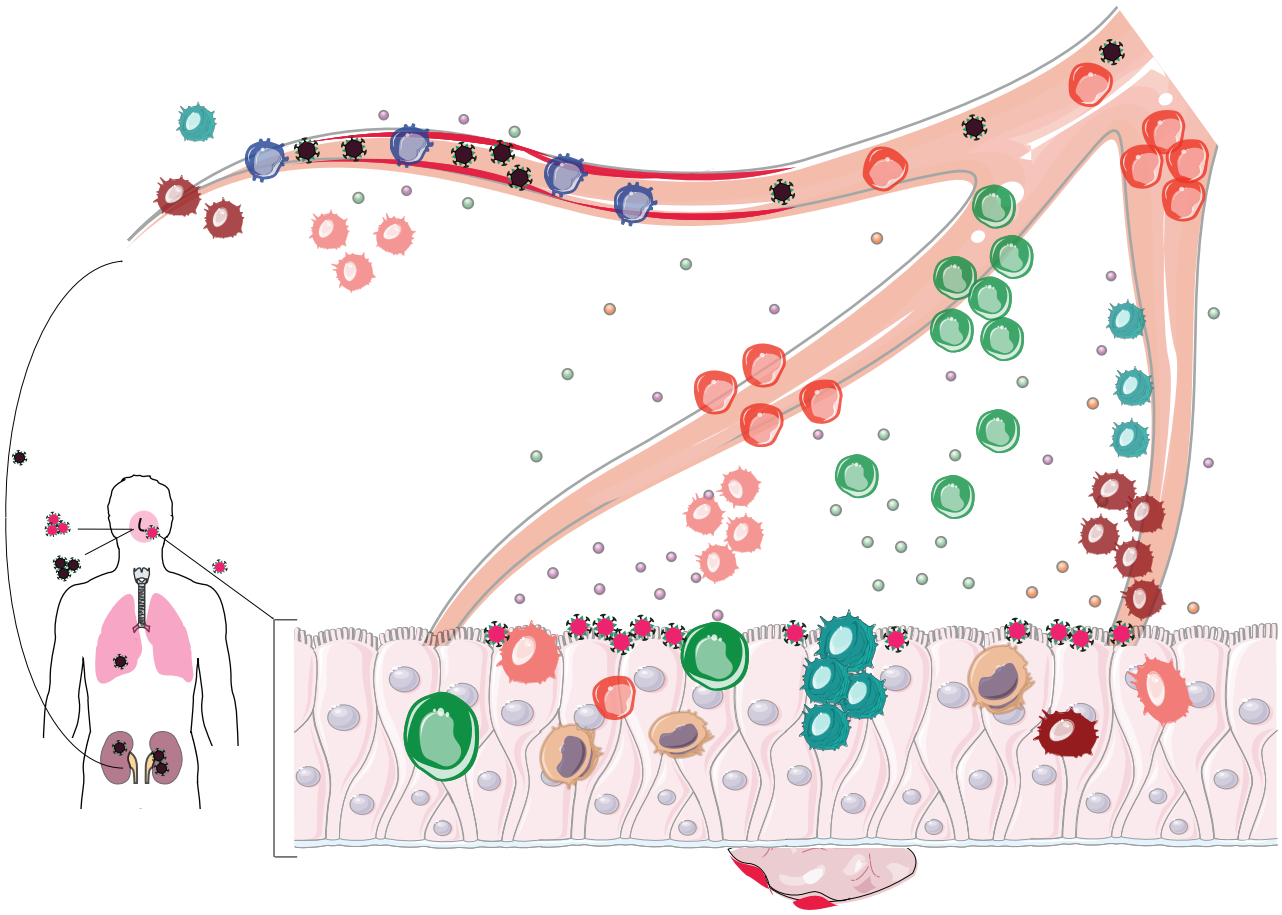
Thesis for doctoral degree (Ph.D.)
2019

Monocytes and dendritic cells: roles during human influenza and hantavirus infections



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MONOCYTES AND DENDRITIC CELLS: ROLES DURING HUMAN INFLUENZA AND HANTAVIRUS INFECTIONS

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Stockholm 2019

Published by Karolinska Institutet.
Printed by US-AB 2019

ISBN 978-91-7831-582-6

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended in Rolf Luft Auditorium, L1:00
Karolinska University Hospital, Solna
Friday November 22nd 2019 09.00

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“If you follow your bliss, you put yourself on a kind of track that has been there all the while, waiting for you, and the life that you ought to be living is the one you are living. Wherever you are – if you are following your bliss, you are enjoying that refreshment, that life within you, all the time.”

Joseph Campbell

To my family. The one I started with, and the one I gained along the way.

ABSTRACT

Viruses infect a vast majority of the other species on the planet and rarely leave the host undamaged. Most human viruses need to infect an individual to replicate and disseminate, and must contend with the full force of the human immune system. On the host side, immune cells must withstand virus infection, impairment of function and possible death, to recruit other immune cells, train them against the specific weaknesses of the adversary, muster a host, eliminate the threat, and leave behind a legacy of sentinels who maintain immune memory. Host-pathogen interactions are, in many ways, like warfare. In this thesis, we describe an important early aspect of the human immune response to two re-emerging viruses of global importance– influenza viruses and hantaviruses. Both influenza viruses and hantaviruses are pathogens which enter through the respiratory route but cause vastly different diseases. A strong, proinflammatory innate response is mounted against influenza viruses, but clearance of virus is carried out by antigen-specific adaptive responses. On the other hand, hantavirus disease is only recognizable once the host immune response has injured the vasculature and starting the cascade of hemorrhagic events associated with the disease. In both instances, the adaptive responses rely on efficient programming and specification by innate immune cells including monocytes and dendritic cells (DCs). In this thesis, we investigated the different roles played by monocytes and DCs during human influenza A virus (IAV) and Puumala orthohantavirus infections.

We first investigated the functional differences between human plasmacytoid DCs (PDCs) from different tissues in the context of IAV infection. We found that PDCs in blood are more potent producers of the antiviral mediator, IFNα, than tonsil-resident PDCs. Next, we studied the distribution and function of monocytes and DCs in circulation and in the upper respiratory tract during acute influenza disease. We observed that IAV infection resulted in expansion of specialized inflammatory monocytes in circulation which cause inflammation via TNFα. Additionally, monocytes and DCs were recruited to the nasopharynx, where the virus is typically located and we found significant evidence of local TNFα and inflammation. To assess whether monocyte and DC redistribution is also provoked by hantavirus infections, we studied patients suffering from hemorrhagic fever with renal syndrome (HFRS) due to Puumala orthohantavirus. We report a dramatic loss of monocytes and DCs in peripheral circulation during acute HFRS and indications of migratory chemokine signaling. And finally, we assessed the influence of disease severity on monocyte redistribution in acute HFRS. We found that severe HFRS is characterized by depletion of nonclassical monocytes from circulation and impairment of myeloid cell ability to respond to additional TLR stimuli. The findings in this thesis indicate tissue- and pathogen-specific differences in inflammatory behavior of human monocytes and DCs.

LIST OF SCIENTIFIC PAPERS

I. **Sindhu Vangeti**, Jens Gertow, Meng Yu, Sang Liu, Faezzah Baharom, Saskia Scholz, Danielle Friberg, Magnus Starkhammar, Alexander Ahlberg, and Anna Smed-Sörensen.

Human blood and tonsil plasmacytoid dendritic cells display similar gene expression profiles but exhibit differential type I IFN responses to influenza A virus infection.

Journal of Immunology, 2019, 202(7):2069-2081

II. **Sindhu Vangeti**, Meng Yu, Sara Falck-Jones, Sang Liu, Asghar Muhammad, Klara Sondén, Jan Albert, Niclas Johansson, Anna Färnert, Anna Smed-Sörensen.

Human influenza A virus infection recruits monocytes and dendritic cells to the nasopharynx in patients with acute disease.

Manuscript

III. Saskia Scholz, Faezzah Baharom, Gregory Rankin, Kimia T. Maleki, Shawon Gupta, **Sindhu Vangeti**, Jamshid Pourazar, Andrea Discacciati, Jonas Höijer, Matteo Bottai, Niklas Björkström, Johan Rasmuson, Magnus Evander, Anders Blomberg, Hans-Gustaf Ljunggren, Jonas Klingström, Clas Ahlm and Anna Smed-Sörensen.

Human hantavirus infection elicits pronounced redistribution of mononuclear phagocytes in peripheral blood and airways.

PLoS Pathogens, 2017, 13(6):e1006462

IV. **Sindhu Vangeti***, Tomas Strandin*, Sang Liu, Johanna Tauriainen, Jukka Mustonen, Antti Vaheri, Olli Vapalahti, Jonas Klingström, Anna Smed-Sörensen. (*equal contribution)

Depletion of CD16+ monocytes and activation during Puumala orthohantavirus caused HFRS associates with disease severity.

Manuscript

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. **Sindhu Vangeti**, Meng Yu and Anna Smed-Sörensen.

Respiratory Mononuclear Phagocytes in Human Influenza A Virus Infection: Their Role in Immune Protection and As Targets of the Virus.

Frontiers in Immunology, 2018, 9:1521 *(Review)*

II. Marlena Scharenberg, **Sindhu Vangeti**, Eliisa Kekäläinen, Per Bergman, Mamdoh Al-Ameri, Niclas Johansson, Klara Sondén, Sara Falck-Jones, Anna Färnert, Hans-Gustaf Ljunggren, Jakob Michaelsson, Anna Smed-Sorensen and Nicole Marquardt.

Influenza A Virus Infection Induces Hyperresponsiveness in Human Lung Tissue-Resident and Peripheral Blood NK Cells.

Frontiers in Immunology, 2019, 10:1116

III. Carles Solà-Riera, Shawon Gupta, Kimia T. Maleki, Patricia Gonzalez Rodriguez, Dalel Saidi, Christine L. Zimmer, **Sindhu Vangeti**, Laura Rivino, Yee-Sin Leo, David Chien Lye, Paul A. MacAry, Clas Ahlm, Anna Smed-Sörensen, Bertrand Joseph, Niklas K. Björkström, Hans-Gustaf Ljunggren, and Jonas Klingström

Hantavirus Inhibits TRAIL-Mediated Killing of Infected Cells by Downregulating Death Receptor 5.

Cell Reports, 2019, 28(8):2124-2139

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BDCA	Blood dendritic cell antigen
BEC	Blood microvascular endothelial cells
BFA	Brefeldin A
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CM	Classical monocyte
CpG ODN	C-p-G oligodeoxynucleotide
CPT	Cell preparation tube (with sodium heparin)
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBB	Endobronchial biopsy
EBM-2	Endothelial cell growth basal medium-2
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMA	Glycidyl methacrylate
HA	Hemagglutinin (IAV)
HFRS	Hemorrhagic fever with renal syndrome
HPS	Hantavirus pulmonary syndrome
IAV	Influenza A virus
IFN	Interferon
IM	Intermediate monocytes
ISG	Interferon-stimulated gene
KIR	Killer-cell immunoglobulin-like receptor
LAIV	Live-attenuated influenza vaccine
LNHD	Lineage negative HLA-DR (cells)

MALT	Mucosal-associated lymphoid tissue
MCP	Monocyte chemoattractant protein
MDC	Myeloid dendritic cell
MDDC	Monocyte-derived dendritic cell
MEK	Mitogen-activated protein kinase/extracellular signal-regulated kinases
MHC	Major histocompatibility complex
MNP	Mononuclear phagocyte
MYD88	Myeloid differentiation primary response 88
NA	Neuraminidase (IAV)
NALT	Nasopharynx-associated lymphoid tissue
NCM	Nonclassical monocytes
NK	Natural killer (cells)
NP	Nucleoprotein (IAV)
NPA	Nasopharyngeal aspirate
PBMC	Peripheral blood mononuclear cell
PDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
PRR	Pattern recognition receptor
PUUV	Puumala virus
RAB	Ras-associated binding protein
RIG-I	Retinoic acid-inducible gene I
TCR	T cell receptor
TLR	Toll-like receptor
TMC	Tonsil mononuclear cell
vRNP	Viral ribonucleoprotein

1 INTRODUCTION

The human immune system functions as the body's physiological defense system to maintain health and homeostasis. Immune cells are constantly vigilant of the threats from foreign antigens and pathogens that come into contact with the body, both externally and internally. Immune cells, which circulate in blood and line the various mucosal surfaces, recognize and respond to pathogens, educate and establish immune memory, facilitate recall responses in the event of re-exposure, and importantly, discriminate between self and non-self antigens. The human immune system is comprised of many integral components- a wide range of immune cells, receptors, ligands and effector molecules, and importantly, the signaling pathways they use to communicate. Every branch of the immune system has distinct functions, all individually programmed to act synergistically to exert potent protective effects.

Of the different pathogens that infect humans to cause disease, viruses have remained perhaps the most enigmatic. Viruses are inert infectious particles comprising proteins, nucleic acids and lipids which enter a host cell (prokaryotic or eukaryotic) and hijack cellular machinery to self-propagate. Speculations of the existence of viruses, and of their contribution to human disease can be traced back to Louis Pasteur who suspected that rabies was caused by a pathogen smaller than bacilli [1]. Subsequently, "filterable" pathogenic agents were discovered which were then named viruses. The human influenza virus, a pathogen causing millions of infections annually, was isolated only in 1933 [2], over a decade after the 1918 pandemic H1N1 influenza that killed 50 million people [3]. Several decades later, the mechanisms by which the 1918 influenza strain proceeded to cause a global pandemic were starting to be understood [4]. Key among them, aberrantly strong immune responses were observed in individuals who died from infection including severe pulmonary pathology resulting in epithelial damage, pulmonary edema and hemorrhage [5, 6]. Tracing the origin and mechanism of abnormal responses was aided by a better understanding of immune cell biology and function at steady state, that was acquired over this period of time.

During this time, following the 1908 Nobel Prize in Physiology and Medicine, awarded to Ilya Metchnikoff and Paul Ehrlich, "in recognition of their work on immunity", the field of immunology evolved from its nascent stages and became a cornerstone of biological research. The emergence of the field of immunology dates back to seminal contributions from Edward Jenner (1796, small pox vaccination); Ernst Haeckel (1862, phagocytosis); Paul Ehrlich (1877, hematological staining and mast cell discovery); Robert Koch, Louis Pasteur and Emile Roux (1876-78, germ theory of disease, attenuation of virulence and acquired immunity); and Ilya Metchnikoff (1882, phagocytosis and the cellular basis of immunity) [7]. Rapidly, the

complexity of the cells and molecules of the immune system started to be described. Of note, the distinction between innate and adaptive immune responses was made following the discovery of dependency of the “lymphoid” cells on “macrophage” function [8]. The innate immune system, in particular the mononuclear phagocytes (MNPs: coined by Ralph van Furth) were then identified as the sentinels of the immune system [9]. Comprised of monocytes, dendritic cells (DCs) and macrophages, MNPs are capable of surveillance for, uptake of, processing and presentation of foreign antigens, to initiate and orchestrate adaptive responses [10].

In the context of a virus infection, specific and efficient humoral and cytotoxic immune responses are essential to clear the infection, minimize host damage and establish immune memory. Each of the MNP subsets have distinct functions during homeostasis and during viral infection. MNPs also possess the ability to migrate to the site of inflammation [11], and to acquire functions of other subsets [12]. However, MNPs are themselves susceptible to viral infection [13], sometimes facilitating the spread of the virus while compromising MNP functions [14]. Over the course of viral disease and during resolution of infection, MNPs reflect the onset of inflammation, instruction of the adaptive response, control of infection by effector cells, subsidence of inflammation and return to steady state. So, mapping the individual contributions of each MNP subset, across tissues, over time and in relation to one another is an ongoing task. Detailed *in vitro* studies and elegant animal models have made meaningful contributions to this field. Human immunology is following, not too far behind.

In this thesis, we attempted to expand our current understanding of MNP function during viral infection by comparing immune responses of human MNPs found in blood and at the site of Influenza A virus (IAV) infection (**Papers I and II**). Additionally, we investigated MNP behavior during hemorrhagic fever with renal syndrome (HFRS) due to Puumala virus infections by detailed, longitudinal and functional characterization in patients (**Papers III and IV**). Taken together, the findings from this thesis improve our knowledge of monocyte and DC distribution and function in human influenza virus and hantavirus infections.

2 AIMS OF THESIS

The general aims of the thesis were to describe the role of human monocytes and DCs, from blood and the tissues of interest during infection *in vitro* and *in vivo*. We aimed to study how they redistribute, mature, cause inflammation, provide antiviral protection, shape adaptive responses and thereby dictate disease severity in viral infection. The specific aims were as follows:

- To compare how human blood and lymphoid tissue-resident plasmacytoid DCs respond to IAV exposure *in vitro* (**Paper I**).

- To study how seasonal human IAV infection influences monocyte and DC redistribution between blood and the nasopharynx (**Paper II**).

- To study how DCs and monocytes are redistributed in blood and airways during hemorrhagic fever with renal syndrome (HFRS) (**Paper III**).

- To assess how severity of HFRS influences the kinetics of monocyte redistribution and activation during HFRS (**Paper IV**).

3 RESPIRATORY VIRUSES

Respiratory infections, especially of the lower tract, contribute significantly to global disease burden and cause an estimated 2.37 million deaths annually [15], with acute respiratory virus infections being the second largest contributor to this number. Mortality is especially high in children under the age of 5 years and adults over the age of 70 years. Respiratory virus infections are associated with increased risk of hospitalization and premature mortality. For children, survival of respiratory viral infection may be associated with increased risk of chronic respiratory diseases like asthma and chronic obstructive pulmonary disease (COPD) during adulthood [16]. Many respiratory viruses pose a global threat, on account of high mutation rate, pandemic potential, rapid transmission, lack of effective vaccines and antivirals, partial vaccination coverage and very often, lack of accurate numbers from areas of high risk and incidence [17]. The human influenza viruses are a perfect example fitting all the above parameters, primarily causing upper respiratory tract infections [18]. Of the millions of cases of seasonal influenza infections, only a small proportion of patients have lower airway involvement, often in the form of primary viral or secondary bacterial pneumonia [19]. Hantaviruses, on the other hand, are also transmitted via the respiratory route. But in addition to causing (lower) respiratory tract symptoms, hantaviruses can have broader effects on the circulatory system and the kidneys [20]. This thesis includes papers on both influenza and hantavirus infections in humans.

3.1 INFLUENZA A VIRUS (IAV)

Influenza, or the “flu”, caused primarily by Influenza A virus (IAV), results in over 650,000 deaths annually [21]. This number is significantly elevated during a pandemic like the Spanish flu (1918-19), which led to about 50 million deaths. Due to the global disease burden, and the pandemic potential of IAV; influenza infections are carefully monitored by the WHO Global Influenza Surveillance and Response System (GISRS), CDC (USA) and ECDC (Europe). In Sweden, Folkhälsomyndigheten (Public Health Agency of Sweden) carries out sentinel surveillance of influenza.

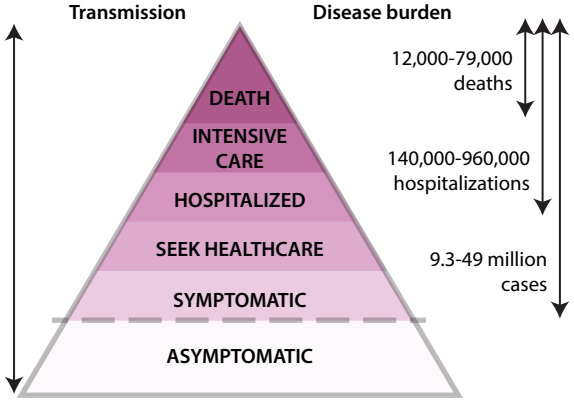

Figure 1. The Influenza disease burden. The clinical manifestation of influenza disease spans a broad range. The virus remains transmissible at every stage. The CDC estimates that since 2010, influenza has caused between 9.3 and 49 million cases in the US alone. Around 10% of these cases were hospitalized and a little under 1% succumbed to influenza.

During a typical influenza season, only a fraction of the population exposed to the virus develop symptomatic disease. The manifestation can range from mild, subclinical infections to severe lower respiratory tract disease and can in some cases be fatal (**Figure 1: Influenza disease burden**) [22, 23]. Complications and severe disease are likely in young children, the elderly and the immunocompromised [22, 24].

Influenza A virus- biology and pathogenesis

Influenza viruses belong to the family *Orthomyxoviridae*, a family of viruses with segmented negative-sense, single-stranded RNA genomes. Influenza viruses A, B and C cause influenza in vertebrates, including humans. IAV causes the majority of human influenza infections, and uniquely, possesses pandemic potential, due to its segmented genome, diverse host range and high mutability [25]. The IAV genome is comprised of 8 segments, coding for up to 16 structural and nonstructural proteins, and other accessory proteins expressed in a strain-specific manner [26, 27].

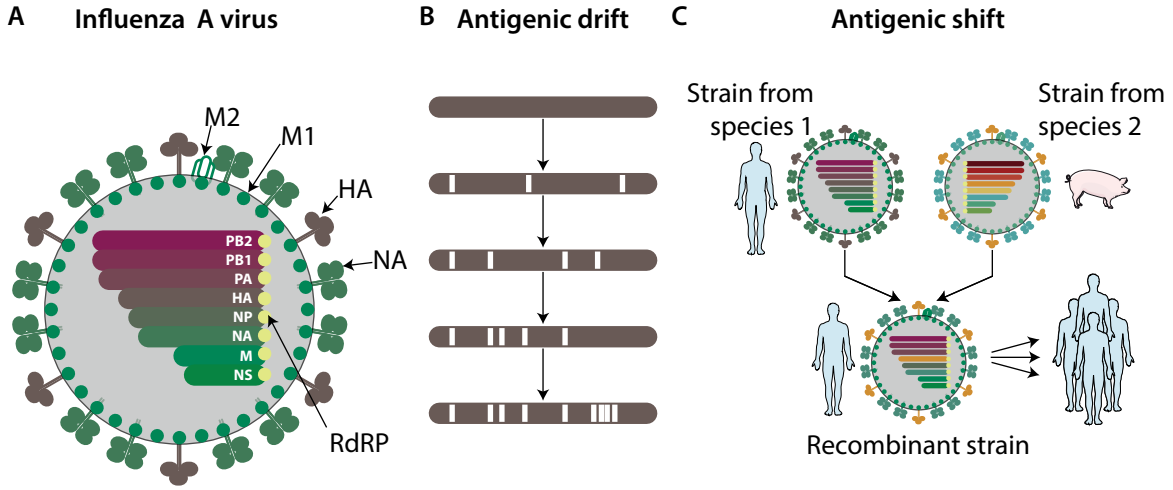

Figure 2. Influenza A virus: Genome organization and antigenic variability. (A) The influenza A and B virus particles are comprised of a ribonucleoprotein core (8 RNA segments associated with the RNA-dependent RNA polymerase, RdRP) in a matrix protein shell (M1). The lipid envelope (host-derived) displays the hemagglutinin (HA) and neuraminidase (NA) glycoproteins that determine host specificity. (B) Antigenic drift is a result of gradual accumulation of single amino acid mutations in HA or NA over time for immune evasion. (C) Antigenic shift is often a result of recombination of viruses from two different species (e.g. human and swine IAVs) in a single host, resulting in a new HA-NA configuration. The recombinant strain can acquire differential sialic acid specificity, enhancing its pathogenicity in humans. Lack of pre-existing immunity can contribute to greater transmission via lack of herd immunity to recombinant strains.

IAV virions can range from 80-120nm sized spherical particles to almost 20µm long filaments (**Figure 2A: Influenza A virus**) [28]. The lipid membrane (host-derived) is studded with spikes of hemagglutinin (HA), neuraminidase (NA) and the matrix protein, M2. The RNA segments are wrapped in nucleoprotein (NP) and the polymerase, forming the viral nucleoprotein (vRNP) [27, 29]. The nomenclature of the virus strains is derived from the combination of H (16 subtypes) and N (9

subtypes) proteins displayed on the virus surface; i.e. H1N1 or H3N2. Point mutations from the lack of proofreading by the RNA polymerase and selection pressure from host immune responses gradually lead to diversity in IAV HA and NA, referred to as antigenic drift (**Figure 2B: Antigenic drift**). The segmented nature, and the wide host range of IAV can lead to reassortment events between two different strains, in the same host cell, resulting in a new subtype, referred to as antigenic shift (**Figure 2C: Antigenic shift**). Additionally, the first exposure in life to influenza can imprint the immune response and prime responses during subsequent exposures, the phenomenon referred to as “original antigenic sin” [30-32]. Pandemic, and especially seasonal influenza strains vary between consecutive episodes/seasons necessitating annual updates to vaccine formulation [24, 33, 34].

Transmission and replication

IAV is spread via inhalation of aerosols and droplets containing the virus [35]. The virus preferentially infects respiratory epithelial cells, remaining localized to the airways [36]. Viremia is exceedingly rare although the absence of the virus does not impede systemic inflammatory effects [37]. The HA of human IAV strains recognizes α(2,6) linked sialic acid residues on host cell glycoproteins and glycolipids[26], primarily in the upper respiratory tract. Avian influenza viruses are preferential to the α(2,3) linked sialic acid residues which are present only in the lower respiratory tract, making humans susceptible to avian strains in the rare event of exposure [38]. The NA cleaves the virus free from HA-sialic acid binding, facilitating uptake via multiple endocytic mechanisms. The virus is trafficked to the early endosome (pH ~5) where HA undergoes conformational changes leading to viral envelope fusion with host cell membranes, releasing the vRNP into the cytoplasm where it is trafficked to the nucleus via nuclear localization signals on NP and host importins [39]. Complementary RNA is transcribed, and with that as a template, copies of new viral RNA are transcribed. Viral mRNA transcription is initiated with cap-snatching by PB2, new transcripts are synthesized and polyadenylated, and translated to viral proteins. New virion assembly occurs at lipid rafts and is followed by budding of virions [27].

Innate immune responses to IAV

Immune responses against IAV span the breadth of the immune system, engaging phagocytes, antigen presenting cells (APCs), cytokines, cytotoxic lymphocytes and antibody-mediated protection. IAV induces a potent innate immune response, required for establishment of adaptive responses; but innate responses also contribute to disease severity. The factors determining the delicate balance between pathologic and protective responses are still poorly understood. IAV, and its components, are sensed via pattern recognition receptors (PRR) like toll-like receptors (TLR- TLR7, TLR8, TLR3) [40], c-type lectin receptors (CLR-CD206,

CD301, DC-SIGN/CD209) [41], NOD-like receptors (NLR- NLRP3, NLRC2, NLRX1) and RIG-I like receptor (RLR) [42] (**Figure 3: Endosomal TLR signaling**). Recognition of IAV triggers intracellular signaling pathways which lead to cytokine production, and importantly, IFN-mediated antiviral signaling (MXA, ISG-15, PKR, IFITM etc.) [43]. The cytokines (and chemokines) recruit innate cells to the site of infection.

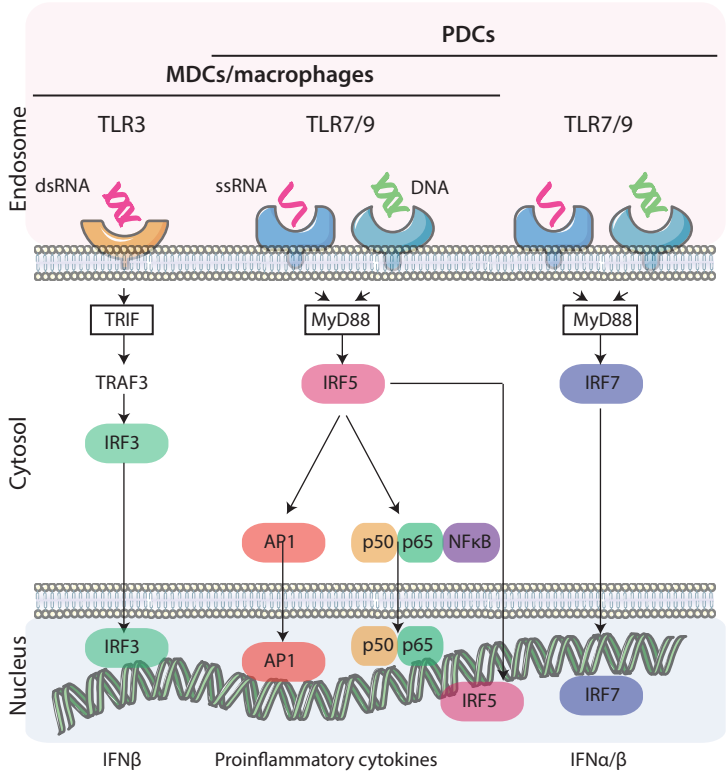

Figure 3. Endosomal TLR signaling. DCs and macrophages sense viruses through endosomal TLRs 3, 7 and 9. Monocytes express TLRs 4, 7 and 8. IAV, being a single-stranded RNA virus, can be recognized via TLR7. Double stranded RNA intermediates are sensed by TLR3 and While TLR3 relies exclusively on TRIF and IRF3 signaling, TLRs 7 and 9 rely on MyD88, IRF5 and IRF7. In PDCs, MyD88 and IRAK4 induce transcription of IFNα/β and subsequent interferon-stimulated gene signaling.

Neutrophils are one of the first cells recruited to the nasopharynx, where they support macrophages in clearing apoptotic, virus-infected cells [44]. However, exaggerated secretion of reactive oxygen species (ROS) by neutrophils can be toxic [45]. Neutrophils can also perform antigen presenting functions to some extent, via MHC II presentation to memory CD4 T cells [46]. Neutrophils also augment the adaptive immune response by leaving trails of the chemokine CXCL12 for CD8+ T cells to the lungs [47]. Recently, however, it was shown that a subset neutrophils can also suppress T cell-mediated lung injury via CD11b/CD18 [48]. As important as neutrophil functions are during IAV infection, they are insufficient to clear infection. The adaptive response capable of clearing IAV infection requires professional APCs.

In addition to neutrophils, innate lymphoid cells (ILCs) offer a potent layer of innate protection. Natural killer (NK) cells, now reclassified with ILC1s, are cytotoxic lymphocytes capable of responding to virus exposure by secreting granzymes, perforins, IFNα and TNFα, to kill infected cells and enhance the function of MNPs recruited to the site of virus infection [49]. NK cells are recruited to the lungs in an IL-15-dependent manner [50], recognize IAV through natural cytotoxicity receptors and

lyse virus-infected cells [51]. Lung resident NK cells in particular, can be primed by IAV to degranulate, secrete TNFα, become sensitive to respiratory epithelial cells and also increase lung-homing capacity [52]. The killer-cell immunoglobulin-like receptor (KIR) repertoire of NK cells dictates their function and contributes to severity of IAV infection [53, 54]. The focus of this thesis, however are the MNPs and their individual contributions to the host response to IAV, which are discussed in greater detail in the next chapter.

Human influenza disease

Influenza infections initially present within 24-48 hours of exposure with symptoms like fever, muscle ache, headache, persistent cough, nasal congestion, sore throat and fatigue [55]. Diagnosis is confirmed by highly sensitive and rapid RT-PCR on nasal or nasopharyngeal samples [56, 57]. Disease management involves control of fever and providing symptomatic relief, and when necessary, antibiotic treatment for secondary bacterial infections. Severe disease follows the spread of the virus to the lower airways, infecting the type II pneumocytes. Diffuse alveolar damage, infiltration of immune cells and a “cytokine storm” mark the development of acute respiratory distress syndrome (ARDS) [19, 58]. Therapeutic interventions are primarily supportive, and aimed at managing the complications (e.g. antibiotics or oxygen support). Antivirals targeting the neuraminidase function of the virus (by competitive inhibition) like Oseltamivir are recommended within 48 hours of symptoms in risk groups [59]. Amantadane compounds, which inhibit the virus (only IAV) by blocking the M2 ion channel function, have been used previously, but are no longer recommended due to side-effects and emergence of drug-resistant viruses [60]. A cap-dependent endonuclease inhibitor drug, Baloxavir, was recently licensed for use in influenza [61], however, strains with reduced susceptibility to the drug are already being reported [62]. The most effective and promising precautionary strategy continues to be prophylactic annual vaccination [63].

Global health implications of influenza

Influenza virus has been causing pandemic infections dating as far back as 1580 [64]. Two more pandemics followed in the 18th (1729 and 1781) and 19th (1830 and 1898) centuries, with the 20th century witnessing 4 influenza pandemics (1918, 1957, 1968 and 1977). Cumulatively, all the influenza pandemics may have caused fewer deaths than seasonal influenza in the last 100 years (**Table 1: Influenza pandemics documented**) [3, 4, 65]. The morbidity associated with influenza (disability-adjusted life years, healthcare costs, hospitalization, exacerbation of comorbidities etc.) incurs a significant burden globally [66, 67]. Of course, estimates are based on long-term epidemiological data and in many developing nations, surveillance data is unavailable, which indicates that the actual numbers may be higher than reported [18]. The largest contributors to disease burden in humans are the influenza viruses A and B, although influenza C viruses have caused symptomatic disease and

hospitalizations in children under the age of 6 [68]. In turn, the largest contribution to influenza transmission comes from infected children with no or weak preexisting immunity to influenza, highlighting the need for targeted vaccination in this risk group [69]. Elderly individuals are also vaccinated in a directed manner but B cell responses are poorly adapted in this population, which can lead to incomplete protection [70].

Table 1. Influenza pandemics documented

Year	Name of the pandemic	Virus subtype	Location first identified	Case fatality rate	Estimated number of deaths
1889-1890	Asiatic/Russian flu	H3N8	St. Petersburg, Russia	0.1- 0.28%	1 million
1918-1920	Spanish flu	H1N1	Kansas, USA	2%	20-50 million
1957-1958	Asian flu	H2N2	Guizhou, China	0.13%	1-1.5 million
1968-1969	Hong Kong flu	H3N2	Hong Kong SAR	<0.1%	0.75-1.0 million
2009-2010	Swine flu	H1N1/09	Mexico	0.03	18000-250000

Influenza vaccines

In the field of vaccine immunology, the development of a universal influenza vaccine has been compared to the holy grail [71]. Our understanding of virus evolution, determinants of protection, dynamics of innate and adaptive response to immunization and the durability of vaccine responses- all remain incompletely understood despite decades of tireless research on each of these aspects. Vaccine design has moved from crudely inactivated viruses or egg-attenuated vaccines, to nanoparticle based vaccines designed in a structure-guided manner (**Table 2: Current and future influenza vaccines**) [63]. Currently, three types of influenza vaccines are licensed for use- inactivated (whole/subunit), live attenuated and recombinant (HA) vaccines. Since the vaccine strains are predictively decided by the WHO several months before the start of the influenza season, there can be a mismatch between the vaccine composition and the strains in circulation. Live-attenuated influenza vaccines (LAIV) are promising, as the cold-adapted nature of the strains prevents replication in the lower airways. LAIVs do not induce efficient seroconversion in adults, but do so in children, inducing high serum HA neutralization titers and mucosal IgA titers [72, 73]. LAIVs are also capable of inducing anti-NA titers, which have been shown to independently correlate with protection [74]. Moreover, LAIVs can be employed as a model system to mimic natural IAV infection as an alternative to human challenge models, and study immune responses in a controlled manner [75, 76].

Table 2. Current and future influenza vaccines

Vaccine type	Target	Breadth of response	Durability	Recommendations
Recombinant vaccine	HA (strong)	+	Short-lived	18-49 years of age
Split or subunit vacine	HA (strong) NA (weak)	+/–	Short-lived	6-12 months of age onwards
Whole virus inactivated vaccine	HA (strong) NA (moderate)	+/–	Likely short-lived	6-12 months of age onwards
Live-attenuated influenza vaccine	HA (moderate) NA (weak)	+	Moderate	Over 2 years of age
Next-generation influenza vaccines	**Antibody-based vaccines** HA stalk domain (headless HA/chimeric HA) HA head domain NA head domain M2 ectodomain		**T cell-based vaccines** NP M1	

Human challenge studies with Influenza virus

Experimental influenza infections in carefully monitored settings have been performed previously with influenza virus, reviewed in [77]. Most of these studies were conducted to assess protective effects of immunization [78-82], to test efficacy of antivirals [83-85] and therapeutic monoclonal antibodies [86]. Only a handful were performed for understanding the pathogenesis of disease [87-89] or to better describe correlates of protection [74, 90-93]. More human challenge studies would rapidly and efficiently advance knowledge of immune function and dysfunction during influenza infections, but safety concerns with using wild-type, replication competent influenza viruses are valid, given their high and spontaneous mutability. Additionally, such studies would also be somewhat limited in scope by difficulty in accessing different compartments of the human respiratory tract. Recently, a wild-type IAV human challenge model (H1N1pdm09 strain) was performed safely [94]. Moving forward, this strategy will prove critical in addressing the global health concern of influenza [95].

3.2 HANTAVIRUSES

Hantaviruses are rodent-borne viruses transmitted to humans via inhalation of dried rodent excrement or saliva containing aerosolized virus. In humans, hantaviruses cause two distinct diseases- hantavirus pulmonary syndrome (HPS), caused by new world hantaviruses like Sin Nombre (North America) or Andes (Latin and South America); and a milder hemorrhagic fever with renal syndrome (HFRS), caused by old world hantaviruses Seoul (global), Hantaan (Asia), Puumala or Dobrava (Europe) (**Figure 4: Geographical distribution of hantaviruses**) [96-98]. Hantaviruses are a growing threat to public health with no therapeutics or licensed vaccines available.

Both HPS and HFRS are characterized by endothelial dysfunction and vascular leakage [99, 100]. In Northern Europe, Puumala virus (PUUV) is the primary cause of HFRS, although different strains circulate during different outbreaks [101]. While hantaviruses have likely caused HFRS for a long time, it was not until an epidemic illness of HFRS during the Korean war (1950-53), that the disease, named Korean hemorrhagic fever after the Hantaan river in Korea, became known worldwide [102]. The isolation of the virus in 1976 by Ho-Wang Lee and subsequent identification of a rodent host led to systematic analysis of hantavirus pathogenesis [103].

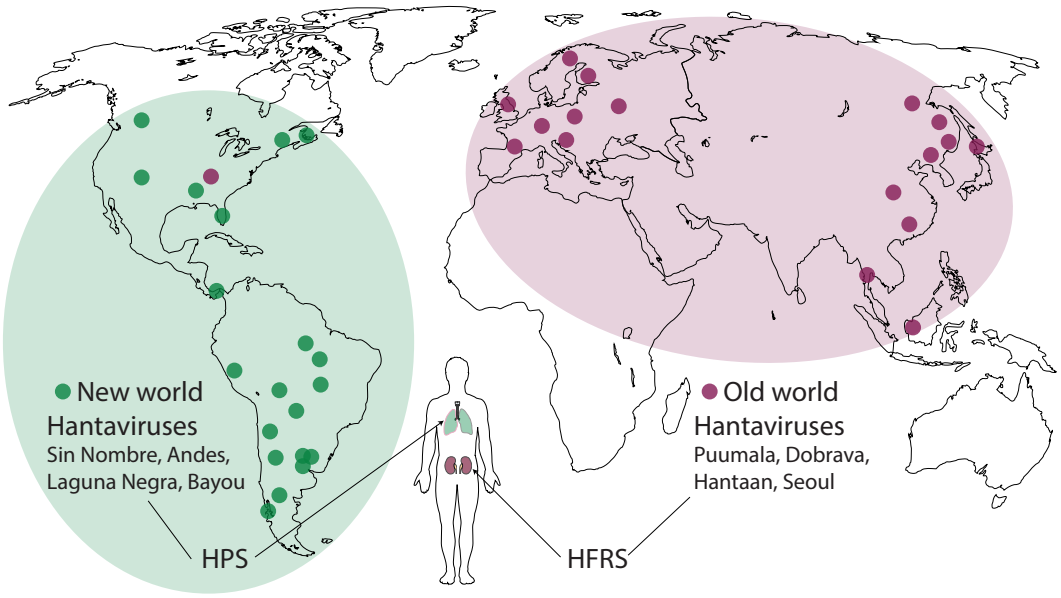

Figure 4. Geographical distribution of hantaviruses. Hantaviruses have a distinct geographical pattern, as do their reservoir hosts. HFRS-casuing "Old world" hantaviruses are found in Europe (Puumala or Dobrava) and Asia (Hantaan or Seoul). The disease has significant renal involvement. HPS-causing "New World" hantaviruses are found in the Americas, and include the Sin Nombre and the Andes viruses. HPS disease manifestation includes cardiopulmonary symptoms and epidemics with high case fatality rates have been reported.

Puumala virus was isolated from a bank vole in Finland in 1980 [104]. The north American Sin Nombre virus was the first HPS-causing hantavirus isolated (1993, Four Corners outbreak) [105]. Compared to influenza, the field of hantavirus biology is still in its nascent stages. The annual disease burden of hantaviruses is considerably lower than that of influenza, causing 150,000 to 200,000 cases [106]. However, epidemics can have high case fatality rates (up to 15% for HFRS and up to 40% for HPS) [20]. The aerosol-based mode of transmission, virus stability, epidemic nature of disease and the lack of vaccines and therapeutics identify hantaviruses as re-emerging zoonotic pathogens of global importance.

Hantavirus- biology and pathogenesis

Hantaviruses belong to the family *Bunyaviridae*, a family of viruses with negative-sense, single-stranded tri-segmented RNA genomes [107]. The three genome

segments named for size as small, medium and large segments, encode the nucleocapsid (N) protein; the glycoprotein precursor (GPC) of structural glycoproteins Gn and Gc; and the RNA-dependent RNA-polymerase (RdRp) respectively (**Figure 5: Hantavirus structure**). Most rodent-borne hantaviruses contain an overlapping reading frame in the small segment encoding the nonstructural protein NSs, with weak IFN-inhibitory function [108]. Hantaviruses were thought to have spherical shape (about 120-160nm in size) with tetrameric glycoprotein spikes, although recent cryo-EM analysis revealed the pleiomorphic nature of the virion structure [109-111]. Hantaviruses are also exceptionally stable at room temperature for several days, aiding their transmission to humans [112]. Endothelial cells are the primary target cells of hantaviruses, where they replicate without inducing cytopathic effects [113]. Integrins are candidate receptors for hantavirus attachment from data generated in *in vitro* models, but *in vivo* confirmation is pending [114, 115]. Recently, new world hantaviruses were shown to use protocadherin-1 for entry in human endothelial cells i*n vitro* [116].

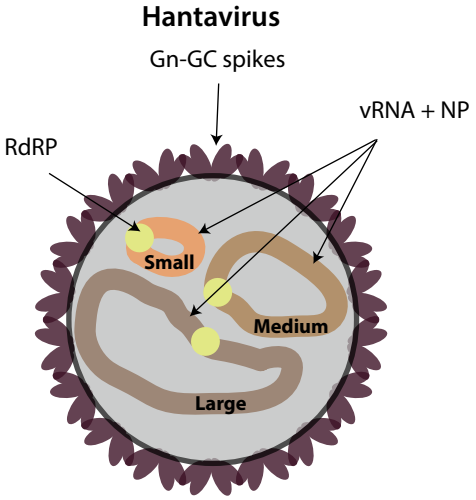

Figure 5. Hantavirus structure. Hantavirus particles are predominantly spherical in shape with some reports of pleiomorphic virions. The lipid envelope is covered in glycoprotein spikes (4 Gn and 4 Gc proteins per spike). The capsid contains the three genome segments- small, medium and large, each segment encapsulated by the N protein. Hantaviruses are quite stable compared to other enveloped viruses, surviving for over 10 days at room temperature. The prolonged survival outside a living host enables transmission which is typically via inhalation of aerosols containing the virus, and not via an arthropod vector like other Bunyaviruses.

Immune response to hantaviruses

Compared to IAV, hantaviruses are less well-studied and the details of the immune response are poorly understood. Hantaviruses are known to trigger an exaggerated cytotoxic lymphocyte (CTL) response [106, 117]. While CTLs are also increased in BAL, hantavirus infected-endothelial cells do not undergo CTL-induced apoptosis [99, 100, 117, 118]. CTLs accumulate in the kidneys during acute PUUV-associated HFRS, and the airways during fatal HPS [119, 120]. Inflammatory cytokines like TNFα and IL-6, secreted by innate cells like monocytes, DCs, macrophages or NK cells, have been reported in serum, urine and tissues of HFRS patients [121-124]. During hantavirus infection, NK cells rapidly proliferate and stay elevated well into convalescence, maintained so by IL-15 which also induced NK cell-mediated killing of uninfected bystander cells [125, 126]. Notably, hantaviruses have differential type I IFN responses, which in turn influence pathogenicity; with delayed IFN and MxA induction facilitating virus replication and dissemination [127, 128]. Hantaviruses can

also induce type III IFN responses, in a type I IFN-independent manner [129]. Despite IFN signaling, and the ability of MxA to bind the N protein, hantaviruses still disseminate [130]. Unlike IAV, hantaviruses cause significant viremia and the virus can be detected in many tissues [131]. DCs in the airways, one of the first migratory cells to come into contact with the virus, have been suggested as a mechanism of spread [132].

Hantavirus disease in humans

Hantavirus manifests as two clinical diseases, HFRS and HPS, with distinct and shared features. Thrombocytopenia and vascular leakage are hallmarks of both diseases [100]. The hemorrhagic component of the diseases localizes in the cardiovascular system in HPS, progressively causing respiratory distress, pneumonia, hypotension and cardiopulmonary shock [133]. During HFRS, increased vascular permeability causes internal bleeding which manifests as petechiae, hypotension or even disseminated intravascular coagulation, and finally leading to kidney dysfunction [134, 135]. The incubation time for the virus can be between one and six weeks. The progression of HFRS is typically febrile, hypotensive, oliguric, polyuric and convalescent, although clinical and laboratory parameters rarely help identify the phase [96, 136]. Renal dysfunction in HFRS causes proteinuria and microscopic hematuria [137]. Pathological findings in the kidney include tubulointerstitial nephritis with immune cell infiltration (neutrophils, monocytes, macrophages, lymphocytes and plasma cells) [138]. Currently, there are no licensed vaccines for hantaviruses. A DNA vaccine based on the M segment of Hantaan and Puumala viruses, administered intramuscularly via electroporation, has demonstrated good seroconversion in a phase I clinical trial [139].

4 MONOCYTES AND DENDRITIC CELLS (DCs)

The following section details the function of mononuclear phagocytes: macrophages, monocytes and DCs. In particular, the biology of the major monocyte and DC subsets, and their functions in the immune response to IAV and hantaviruses will be discussed.

4.1 MONONUCLEAR PHAGOCYTES (MNPs)

MNPs play important roles at mucosal barriers and serve dual purposes of protection and tolerance [140, 141]. MNP subsets possess varying capacity of antigen uptake, processing and presentation abilities and regulate adaptive responses differently [10]. Significant knowledge has been gained from studies on MNPs in mouse models, allowing for careful delineation of their ontogeny and function [142-145]. The ontogeny of human blood MNPs, often clouded by the great plasticity these cell subpopulations share, is being understood with the help of detailed studies on fate-mapping animal models [146-148] complemented with radical studies in humans [149]. The distribution of these cells in human tissues, including the respiratory tract, is also being revealed, along with characterization of their local functions [150-160].

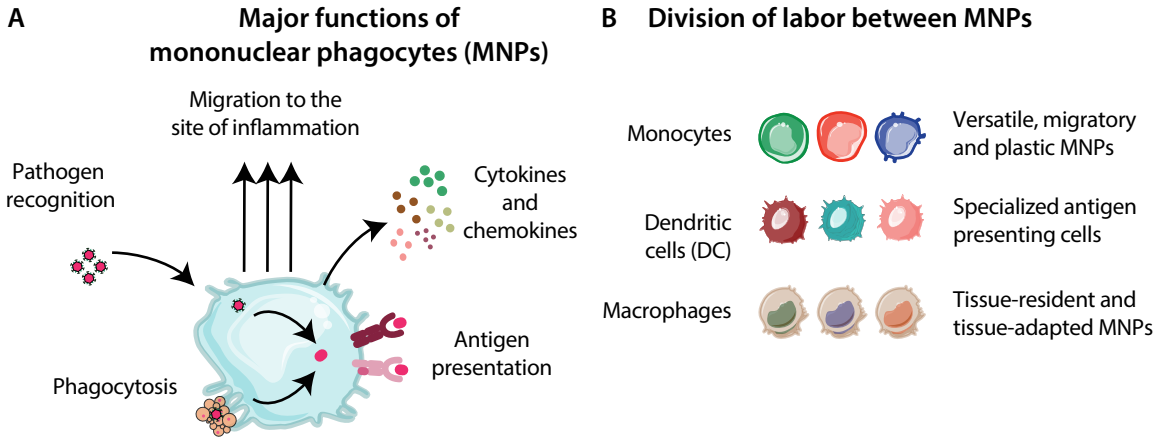

Figure 6. Overview of MNP functions. (A) Mononuclear phagocytes (MNPs) perform diverse roles as the sentinels of the immune system. MNPs can recognize pathogens via PRRs (TLR/NLR/RLR/CLR) and phagocytose the pathogen and debris from dead cells. MNPs also attract other immune cells to the site of the pathogen entry via cytokine and chemokine signaling. MNPs from blood can migrate to the site to kill the pathogen, transport the antigen to the lymph node to process and present antigens, and educate T and B cells. (B) The term MNP refers to three distinct cell types- monocytes (classical, intermediate or nonclassical), dendritic cells (CD1c+ or CD141+ myeloid and plasmacytoid), and macrophages (alveolar, microglia, Kupffer cells etc.). Each MNP excels at a function, and each monocyte/DC/macrophage subset is further specialized.

The major MNP types have characteristic features- macrophages are excellent at phagocytosis; monocytes can rapidly migrate towards inflammation and produce a variety of inflammatory cytokines and chemokines; while DCs are excellent antigen presenting cells and uniquely, possess the ability to activate naïve T cells (**Figure 6: Overview of MNP functions**). In the human immune system, two major groups of

DCs and three distinct monocyte subsets have been identified (in blood). MNPs do not express any lineage markers (CD3, CD19, CD20, CD56 or CD66) but express high levels of HLA-DR [156, 161-163]. Comparative studies extended this knowledge to identify other (rarer) MNP subsets in blood, and importantly, in different tissues.

Hematopoietic development of MNPs

Monocytes are highly plastic cells and precise combinations of cytokines can [164] differentiate them into DCs and macrophages *in vitro* [165, 166]. *In vivo*, they can rapidly migrate to a site of inflammation and acquire a DC-like or macrophage-like phenotype [167-171]. Therefore, DCs and macrophages were long considered specialized or more differentiated variants of monocytes. Recent transcriptomics studies, however, have identified distinct precursors for DCs (common dendritic cell progenitor, **CDP**) and monocytes (common monocyte progenitor, **CMoP**). Macrophages can arise from embryonic precursors (yolk sac, fetal liver or bone marrow monocytes) early in life, and can maintain during adulthood by self-renewal [145-148, 159, 172-174]. DCs differentiate from CDPs under the control of FLT3 ligand [175]. The exact developmental pathways are being constantly challenged and revised [147] (**Figure 7: Overview of monocyte and DC hematopoiesis**).

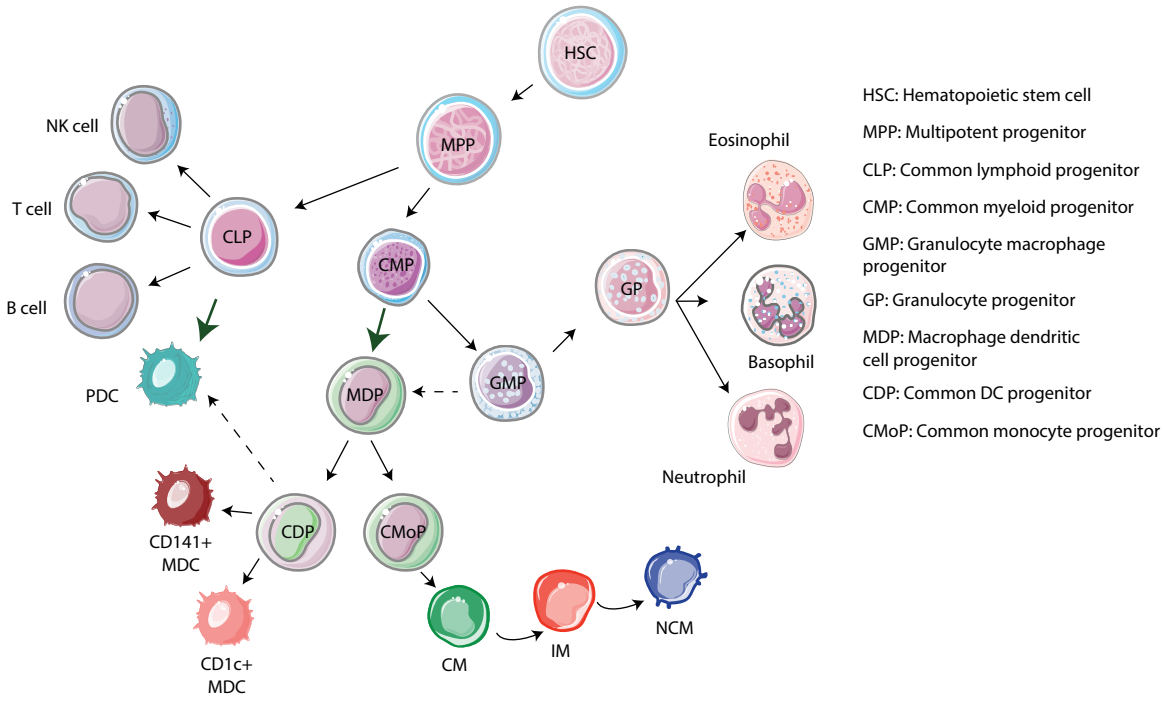

Figure 7. Overview of monocyte and DC hematopoiesis. Monocytes and DCs originate from a common progenitor- the macrophage dendritic cell progenitor (MDP). Recent fate-mapping studies challenged the dogma of all DCs arising from a common DC progenitor (CDP). The PDC lineage can be traced back to the common lymphoid progenitor (CLP). Also, the common monocyte progenitor (CMoP) comes directly from the MDP, independently of the GMP. Classical monocytes (CM) egress from the bone marrow into circulation, where they further sequentially differentiate into intermediate (IM) and nonclassical monocytes (NCM).

Macrophages

Macrophages are tissue-resident professional phagocytes with antigen-presenting capacity. They were first described by Ilya Metchnikoff [84] in starfish larvae, when he observed “phagocytes” trying to surround a rose thorn used to pierce the larva. Since then, macrophages have been found in all tissues where they perform the important function of patrolling for pathogens and clear foreign bodies, pathogens, infected cells and debris [176]. Of all the MNPs, macrophages are the most terminally differentiated. Macrophages also undergo tissue-specification via transcriptional regulation of the core macrophage program before birth that is maintained by tissue microenvironment-specific cues [177]. Macrophage endo-lysosomes efficiently process their contents, with the agenda of degrading the pathogen rather than preserving antigens for presentation to T cells [40, 178]. In the respiratory tract, alveolar macrophages (AM) are the major resident phagocyte population, where they are present in the lower airways, closely associated with alveolar epithelium [179]. Interstitial macrophages are less well-studied, as they are difficult to isolate from their resident tissue, the lung parenchyma [180, 181]. Similarly, macrophage-like cells (CD11b+CD11c+CD14+CD32+CD64+CD68+RFD-7+) have been reported in the nasal mucosal tissue, but remain poorly studied [182]. In severe influenza (highly pathogenic avian strains or human pandemic strains) disease, AMs produce cytokinemia (IFNα/β TNFα, IL-1α/β, IL-6, and chemokines CCL2, CCL4, CCL5, CXCL8 and CXCL9) causing diffuse alveolar damage [183-186]. Macrophages also form large multicellular aggregates of immune cells called granulomas, in chronic infections (*Mycobacterium tuberculosis*) [187] and inflammatory diseases (sarcoidosis) [188].

Monocytes

Monocytes in human peripheral blood are currently grouped into three major categories, based primarily on the surface expression of two markers, CD14 and CD16. Increasing expression of CD16, and decreasing expression of CCR2 on the surface of blood monocytes also marks the differentiation of monocytes after they enter circulation. At steady state, classical monocytes (CM: CD14+CD16+) are the most frequent subset, followed by non-classical monocytes (NCM: CD14-CD16+) and the transient population of intermediate monocytes (IM: CD14+CD16+) [157, 189-191]. Following egress from the bone marrow, CMs remain in circulation for about a day and an estimated 99% extravasate to tissues. The remaining CMs are estimated to first differentiate into IMs (with a lifespan of ~4.3 days), and eventually into NCMs, remaining in circulation for a further 7.4 days [149]. Based on the studies in blood, recent studies have begun to characterize monocyte subsets in healthy human tissues, starting with the respiratory tract, where all three monocyte populations have been identified in the bronchoalveolar lavage, bronchial wash [151], endobronchial biopsies [150, 151], lung parenchymal tissue [153], trachea,

lymph nodes and pulmonary vessels [153] and compared to peripheral blood [154, 156]. The relative distribution of these monocyte subsets is different in each of these compartments. While in blood, CMs are the most frequent monocyte subset, in the airways, often IMs are more abundant, with NCMs being detected in very low numbers [151].

Functionally, monocyte subsets have distinct yet overlapping roles- CMs and IMs are more potent at phagocytosis than NCM. While CMs and IMs have comparable ability to secrete TNFα, CMs are superior at IL-6 and IL-1β secretion [190]. The same study also showed that of the monocyte subsets, only CM were shown to present antigens to T cells *in vitro*, after first differentiating into monocyte-derived DCs. IMs, and to some extent NCMs, are expanded during some infections and inflammation [11, 176, 192, 193]. Most importantly, monocytes possess plasticity that allows them to rapidly differentiate into DCs and macrophages at the site of infection/inflammation [13, 167, 171, 190, 194, 195]. During IAV infections, monocyte subpopulations are also detected in the nasal mucosa [196-198]. NCMs, due to their rarity, have been less studied. NCMs primarily patrol the vasculature, surveil tissues and efficiently recognize viruses through TLR7 and TLR8 sensing, and a unique MYD88-MEK pathway [199-201].

Dendritic cells

Dendritic cell (DC) function is essential to both the innate and adaptive arms of immunity. DCs are by far, the most efficient APCs, with the unique ability to activate naïve T cells. Canonically, DCs take up antigen at the site of infection, become activated, migrate to the lymph node and initiate T cell responses [10, 202]. DCs also efficiently cross-present antigens via MHC I, especially CD141+ MDCs [203]. DC ontogeny has been studied in great detail in mice with detailed functional models and iterative transcriptional analysis helped ascertain human counterparts of the mouse subsets, summarized in (**Figure 8: Homology between human and mouse MNPs**) [203-208].

In the human immune system, DCs are grouped into two clusters: CD11c+ myeloid and CD123+ plasmacytoid DCs (MDCs and PDCs, respectively). MDCs can be further subdivided into two major subsets- CD141+ MDCs (conventional, cDC1) and CD1c+ MDCs (cDC2), with distinct functional advantages [159, 160, 207, 209-211]. The skin has a CD11c and CD1a-expressing resident population of DCs, also referred to as Langerhans cells (LCs). LCs were the first DCs identified and have been studied extensively during inflammatory skin diseases, especially psoriasis [212, 213]. PDCs are present in blood, but more abundant in lymphoid tissues including tonsils [214, 215]. PDC are potent producers of type I interferons which are important in protection against viruses [216-218].

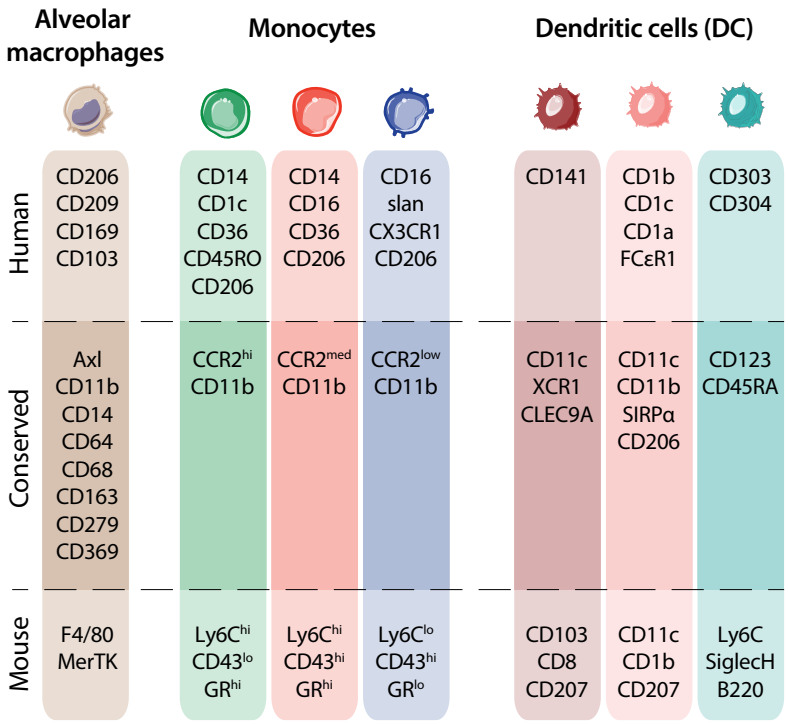

Figure 8. Homology between human and mouse MNPs. Human MNPs have been less well-studied as compared to their murine homologs. The major monocyte and DC subsets, and alveolar macrophages share some markers between the species, however, making it easier to map human DC function based on transcriptional studies in mouse models.

A slan/M-DC8-expressing subset of DCs was also identified in human tonsils, possessing constitutive TNFα-secreting and antigen presenting abilities, but later clarified to be slan+ NCMs [219, 220]. Also from tonsils, a CD207/Langerin-expressing DC subset was identified- possibly differentiated locally from CD1c+ MDCs [221]. Yet another subpopulation of DCs with a distinct phenotypic signature was identified recently, using singe-cell RNA-sequencing, referred to as AXL and SIGLEC6-expressing “AS DCs” [103]. While the transcriptional studies indicate that these subsets of DCs are distinct, all of their (unique) functions need to be identified, which is often complicated by the intrinsic plasticity of DCs during inflammation and infection.

Antigen presentation and adaptive immune responses

The central topic of this thesis has been the antigen presenting cells (APCs), in particular, monocytes and DCs. A major function of these cells is their capacity to process and present antigen to T cells. However, the studies included in this thesis focus on the innate functions of these cells. The following paragraph provides a brief overview of the role of DCs in initiating adaptive immune responses. DCs can take up antigens via phagocytosis, pinocytosis or receptor-mediated endocytosis [222, 223]. DCs possess specialized endo-lysosomal structures which permit optimal processing of protein antigens into peptides rather than rapid degradation. Exogenously-derived peptides are loaded onto MHC class II molecules for presentation to CD4 T cells [224, 225]. In contrast, endogenously expressed proteins

are presented as peptides by MHC class I molecules to CD8 T cells. Peptide presentation via MHC I on APCs is necessary for initiating the cytotoxic effector cells needed to clear viral infection [226, 227].

Additionally, some APCs can present exogenously acquired antigens on MHC I via the cross-presentation pathway [228], enabling APCs to present viral antigens (e.g. IAV) without being infected themselves. Human blood CD141+ MDCs are able to efficiently cross-present antigens derived from necrotic cells [203, 205]. In the context of viral infection, CD141+ MDCs can derive antigen from infected bystander cells or even CD1c+ MDCs (which are comparatively more susceptible to infection) [229]. In line with the overlapping nature of DC functions, the ability to cross-present antigens, is not restricted to the CD141+ MDCs. Human tonsil CD1c+ MDCs and PDCs were also shown to possess cross-presenting capacity [230]. However, recent high resolution studies on pure sorted human PDCs revealed antigen presentation to be a function of pre-PDCs (expressing CD11c, similar to MDCs) and not *bona fide* PDCs [159]. CD1c+ MDCs can also present lipid antigens via CD1a, CD1c and CD1d, activating αβ and γδ naïve T cells and NKT cells [231, 232]. Moreover, CD1c+ MDCs polarize the T helper cell response by secreting the optimal cytokines for individual Th subsets (Th1/Th2/Th17 etc.) [233].

T cell priming and polarization by DCs is dependent on 3 signals- MHC-bound peptide recognition by the cognate T cell receptor (signal 1); costimulatory molecules (CD80/CD86) on APC signaling via CD28 (or CTLA-4) on T cells (signal 2); and finally, T helper cell polarizing cytokines (e.g. Th1: IL-12, IL-23, type I IFN or Th2: IL-4, IL-5, IL-9) (signal 3) (**Figure 9: T cell activation by DCs**) [233, 234].

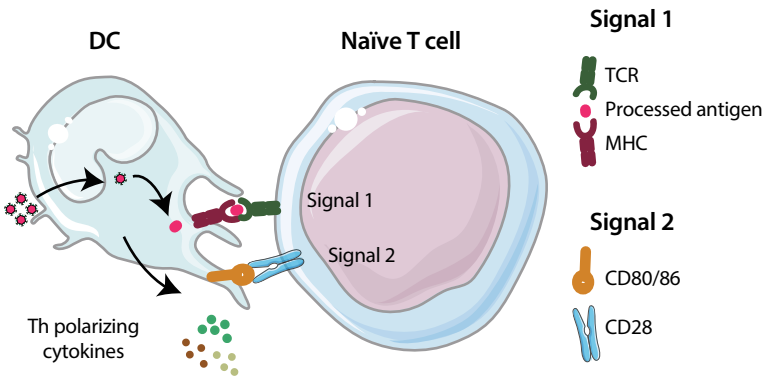

Figure 9. T cell activation by DCs. DCs can process antigens via exogenous or endogenous pathways. The processed antigen is loaded on MHC molecules for presentation to a cognate naïve T cell with the corresponding TCR. CD80/CD86 interaction with CD28 provides the secondary costimulatory signal. Cytokines polarize Th subsets.

4.2 IMPORTANCE OF TISSUE LOCATION

In humans, blood is often the most easily accessible compartment to isolate and study immune cells. A majority of what is known about human MNP function is based on studies done on primary blood DCs or DCs differentiated from blood monocytes in the presence of GM-CSF and IL-4. Several DC and monocyte subsets have been identified in blood and their functions have been studied in great detail

[156, 163, 203, 235, 236]. While there are definite practical advantages to using IL-4 and/or GM-CSF generated monocyte-derived DCs (MDDCs) and monocyte-derived macrophages, they are not a perfect representation of primary DCs or macrophages [237]. Whenever possible, obtaining primary cells from tissues of interest is preferable.

Respiratory tract

The respiratory mucosal surface has a large luminal surface area of approximately 50-100m^2 [238]. It is constantly exposed to the external environment, challenged with environmental factors, allergens and pathogens. The anatomical complexity in different compartments of the respiratory tract is reflected by their respective immune cell composition. Our group and others have previously showed that different immune cells are found in the bronchoalveolar lavage (BAL), bronchial wash (BW), endobronchial biopsies (EBB), lung parenchymal tissue, trachea, pulmonary vessels, nasal mucosa and tonsils as compared to peripheral blood in humans [151, 153, 154, 156, 182, 197, 214]. Depending on the compartment being sampled, several of these DC and monocyte subsets were identified in patients with respiratory infections or disease, but also in healthy individuals (**Figure 10: Sampling sites in the human respiratory tract**). The invasive methods required to sample these locations sometimes make studies on healthy humans methodologically and ethically challenging. In **Papers I and II**, we have used easily-accessible mucosal tissues and well-tolerated sampling methods respectively, to overcome these challenges. However, as in **Papers III and IV**, when respiratory tissues are inaccessible, we must rely on blood and *ex vivo* techniques to perform longitudinal and mechanistic studies.

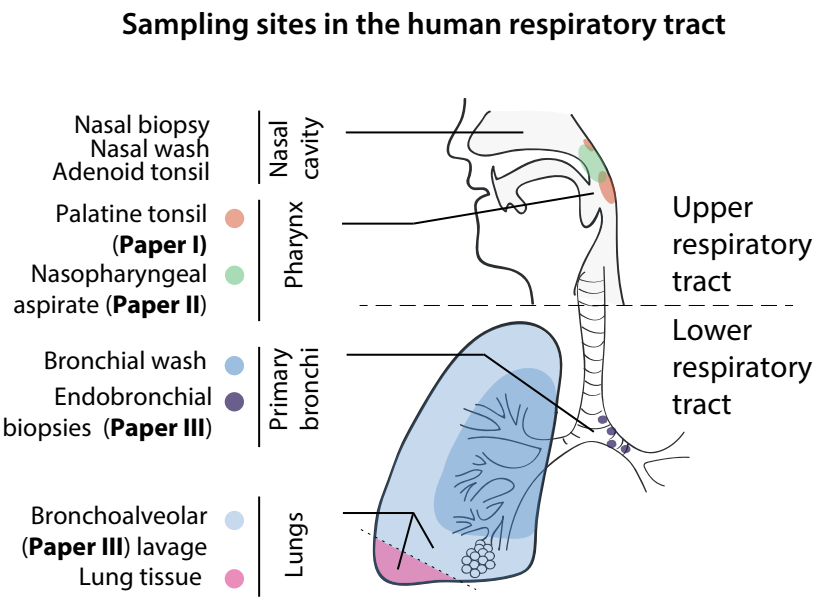

Figure 10. Sampling sites in the human respiratory tract. The upper and lower airways are anatomical compartments with distinct immune cell composition. Routine tonsillectomies and nasopharyngeal aspirates allow sampling of the upper respiratory tract. Bronchoscopies and biopsies are used to sample the lower airways. Although the method is invasive, different branches of the respiratory tree can be concurrently sampled.

Lymphoid tissue

The lymphatic system serves both circulatory and immune systems. Lymph vessels maintain fluid balance by restoring interstitial fluid to blood and lymph nodes act as a sieve trapping pathogens and foreign antigens [239]. It is also the primary site for antigen presentation, lymphocyte activation and proliferation, and generation of an antigen-specific adaptive response. In addition to lymph nodes, the bone marrow, thymus and spleen are considered the primary lymphoid organs. Apart from these, aggregate lymphoid tissue can be found governing the mucosal linings- in the nasopharynx as the tonsils (nasopharynx-associated lymphoid tissue, NALT), in the bronchi (bronchus-associated lymphoid tissue, BALT) and along the gut as Peyer's patches (mucosal-associated lymphoid tissue, MALT) [240]. Of these, the NALT is of the most relevance during an upper respiratory tract infection like IAV [72, 241-243] and we have studied DCs from human tonsils as a source of DCs residing at the site of IAV infection in **Paper I**. The adenoids and the palatine tonsils form a ring of lymphoid tissues, creating an anatomical barrier (Waldeyer's ring) between the nasopharynx and lower airways to prevent pathogen entry, and presumably, act as a source of immune cells [214].

The nasopharynx

The human nasopharynx is the region between the nasal sinuses and the pharynx. The nasopharynx is covered in ciliated epithelium with goblet cells, where the sialic acid distribution is of the α(2,3) type making it a permissive site for IAV replication [240, 244]. As demonstrated in a ferret model, the respiratory epithelium in the upper airways supports IAV adaptation in favor of increased transmissibility [242]. The human nasopharynx can be sampled for immune cells by nasal wash/lavage [198, 245], biopsies [182, 246] or nasopharyngeal aspirates (as we performed in **Paper II**). The nasopharynx has been used extensively, for detection of viruses and soluble markers of inflammation, but few studies report cellular analysis of this compartment. Moving forward, the nasopharynx, especially in the context of influenza, needs to be better studied and utilized for its true potential- a source of longitudinal samples from the site of IAV replication.

Bronchi and lungs

The lower airways of the human respiratory tract have been investigated better than the upper airways, perhaps due to existence of defined sampling techniques. Inflammation or infection of the lower airways are a serious health condition, and require rapid diagnosis. The vasculature of the lung is one of the densest in the human body and as the organ responsible for oxygen supply, lung function is critical for life. Bronchoscopies for exploratory and diagnostic purposes are routinely performed and yield cells from the bronchi and the alveolar space, and can be used to collect biopsies from the bronchial walls and lymph nodes adjoining the airways

[150, 151, 247]. Additionally, lung parenchymal tissue can be obtained from biopsies as non-diseased sections are collected to compare pathology [52, 153, 156]. Bronchoscopies are typically not recommended or necessary during respiratory viral infection, but can be performed during HFRS after reduction of thrombocytopenia, as we performed in **Paper III**.

4.3 SPECIALIZED ROLES FOR MONOCYTE AND DC SUBSETS DURING IAV INFECTIONS

During IAV and RSV virus infections, monocytes and DCs have been proposed to mobilize from peripheral blood to the site of infection, the respiratory mucosa [196-198]. Some of their specialized functions, in the context of IAV infection are discussed below.

Alveolar macrophages: powerful MNPs

In humans, AMs have only been investigated during severe influenza with lower airway involvement (pneumonia or ARDS). AMs can take up IAV by phagocytosing infected alveolar epithelial cells (containing intact virions) and are permissive to replication but produce few productive viral progeny [183, 248, 249]. When *in vitro*-differentiated human AMs are exposed to IAV infection, there is a significant induction of interferons (IFNα and IFNγ), cytokines (TNFα, Il-6, IL-29) and chemokines (CCL2, CCL4, CCL5, CXCL8 and CXCL9) [184, 250, 251]. Alveolar damage in pandemic IAV infections (1918 and 2009) were both characterized by early and excessive macrophage infiltration, generally in the lungs but specifically in the small airways [186, 252, 253].

Classical monocytes: migratory MNPs

CMs are typically the first MNP to rapidly infiltrate the airways during IAV infection from circulation [171, 198]. CMs can phagocytose different antigens and stimulate T cell proliferation [191, 201]. In circulation, TNF-producing CM numbers are elevated (presumably, following an influx from the bone-marrow in a CCR-2 dependent manner) and associate with disease severity [198, 254-257]. *In situ*, monocytes also possess the capacity to differentiate into DCs, especially upon exposure to highly pathogenic IAV strains [171, 194]. During severe influenza, CMs are associated with TNF-mediated pathology in the lungs [258, 259]. As severe influenza is also marked by a hyper-inflammatory response, CM functions may be exaggerated. The extent of CM involvement in milder disease is currently unknown. Additionally, CMs, being susceptible to IAV infection [260, 261], experience functional impairment due to impaired NF-κB and IFN signaling [262, 263]. A weaker IFN response may trigger a feedback loop, resulting in prolonged recruitment of CCR2+ monocytes to the lungs, contributing to tissue damage [256].

Intermediate monocytes: stress response MNPs

IMs, as their name suggests, appear to be an intermediary stage between CMs and NCMs, and therefore, more heterogeneous than the other monocyte subsets. IMs are rapidly expanded in blood during viral infections like dengue [192], RSV [264] and chikungunya [265] or sepsis [193]. IM expansion during acute dengue infection was accompanied by a strong plasmablast response [192]. IMs are also potent producers of cytokines, especially TNFα [266]. During severe IAV infection, they were reported to be increased in circulation and in the nasopharynx [198], but whether milder disease also induces the same pattern, and if the humoral response to IAV is influenced by the IM compartment, is unknown.

Nonclassical monocytes: patrolling MNPs

NCMs are estimated to remain in blood the longest (~7.4 days), of the different monocyte subsets [149]. NCMs also have a distinct motility pattern, whereby they crawl along the endothelial cells [267, 268], and can take up antigens [168, 269]. During homeostasis, it is believed that NCMs function as sentinels in the vasculature. NCMs can respond to TLR7/8 by secreting IL-6, IL-8 and CCL2 in a MyD88-MEK dependent manner [201]. During severe IAV infection, NCMs are present in the nasopharynx, and appear to correlate with local inflammation in an age-dependent manner [198]. Whether they are recruited from vasculature, or undergo differentiation *in situ* (from CMs recruited to the nasopharynx), and if they function to relieve inflammation, remains unknown.

CD1c+ MDCs: versatile APCs

CD1c+ MDCs (also known as cDC2s), are the most well-studied DC subset. CD1c+ MDCs are the most abundant DC in blood [163], have a broad TLR repertoire (TLRs 1, 2, 4, 5 and 8) respond to a variety of pathogens, and can secrete TNFα, IL-10 and IL-1β [270]. *In vitro*, CD1c+ MDCs are susceptible to IAV infection, with impairment of antigen-presenting functions and at higher viral loads, cell death [14, 271, 272]. The initiation of adaptive responses coupled with virus restriction to the tissues suggests APC (likely CD1c+ MDCs) are involved in transporting IAV to the draining lymph nodes. MDCs (CD11c+) and PDCs have been reported in increased numbers in the nasopharynx during IAV infection, but their functional capacity remains to be evaluated [196, 197].

CD141+ MDCs: specialized APCs

Strong CD8+ T cell responses are a hallmark of immune responses in virus infections, which requires presentation of antigen on MHC class I, either as a consequence of infection of the APC and direct presentation or via cross-presentation, where endogenous antigen ends up on MHC I for presentation to CD8+ T cells. CD141+ MDCs (cDC1, expressing CLEC9A or DNGR1), are

specialized DCs with superior ability to cross-present antigens via MHC-I to CD8 T cells [203, 205, 206, 235]. They express TLR3 allowing them to respond to poly(I:C). CD141+ MDCs importantly, are resistant to IAV (and other enveloped virus) infection constitutively, due to specialized endocytic machinery and the high expression of Ras-related protein, RAB15 [229, 273]. CD141+ DCs are less frequent in blood than CD1c+ MDCs, and have never been reported in the nasopharynx before. Interestingly, CD141+ MDCs can also produce type III IFN, which is of special importance in infections where type I IFN cannot fully protect the host [152]. Whether CD141+ MDCs are of greater importance during IAV infection is currently unknown.

PDCs: potent antiviral DCs

PDCs, as described, rapidly produce large quantities of type I IFN in response to TLR7/9 engagement [216, 218, 274-276]. Type I IFN mediate the strongest innate antiviral protection by transcriptionally regulating a multitude of ISGs [250, 277]. A well-studied ISG, MxA (human), is expressed at high levels in PDCs [278-281]. Canonically, blood and tissue resident PDCs have been considered refractory to virus infection, largely due to their elevated, and protective, levels of MxA [229]. Additionally, PDCs (like CD141+ MDCs) also constitutively express RAB15 which restricts viral envelope fusion within the Golgi compartments, conferring these DCs antiviral resistance [229]. *Bona fide* PDCs were also shown to lack antigen-presenting abilities [159], suggesting their principal function during IAV infection may be rapid and large-scale IFNα production. In **Paper I**, we demonstrate that the resistance of PDCs to virus infection *in vitro* can be overcome by high viral load, and similar conditions have been shown to cause rapid apoptosis of PDCs previously [275, 282]. In pregnant women, a group at risk of severe influenza disease, PDCs were reduced in circulation and had attenuated IFNα responses, contributing to lack of protection [283].

4.4 SPECIALIZED ROLES FOR MONOCYTE AND DC SUBSETS DURING HANTAVIRUS INFECTIONS

Our understanding of the cellular innate immune responses to hantaviruses is currently very limited. More is known about the function of NK cells and CD8 T cells in hantavirus pathogenesis, and their contribution to vascular leakage [117-119, 284]. A strong serological response is also seen during acute HFRS [285]. Likely, an innate response must precede the adaptive response. However, only a handful of clinical studies exist on hantaviruses where monocytes from patients have been studied [286-288], although other studies have been performed on patient sera to identify markers of disease severity [124, 285, 289-291]. During Hantaan virus infection, IMs were reported in increased frequencies during acute HFRS, along with increased expression of CD163 and CD206 on IMs [286, 288]. With **Papers III and IV**, we attempt to address this gap in the existing literature.

5 MATERIALS AND METHODS

A brief description of the principal methods and experimental setups used in Papers I-IV are outlined below. Detailed information on materials and methods can be found in the corresponding original papers.

HUMAN SAMPLE MATERIAL

Ethical approval for studies involving humans and human material was granted by (i) the local Ethical Review Board at Karolinska Institutet, Stockholm, Sweden (**Paper I**); (ii) the Swedish Ethical Review Authority (**Paper II**); (iii) the regional Ethical Review Board at Umeå University (**Paper III**); and (iv) the Ethics Committee of Tampere University Hospital (**Paper IV**) respectively. Signed informed consent was obtained in accordance with the Declaration of Helsinki.

BLOOD AND TISSUE SAMPLING

Buffy coats prepared from blood of healthy volunteers were obtained at the Karolinska University Hospital, Stockholm (Sweden) (**Papers I-III**) and the Finnish Red Cross blood service, Helsinki (Finland) (**Paper IV**). Venous blood (EDTA-containing vacutainers, BD) was also obtained from healthy volunteers at the Karolinska University Hospital, Stockholm (**Paper II & III**) and the University Hospital of Umeå, Umeå (CPTs, BD) (Sweden) (**Paper III**).

Tonsils were obtained from patients undergoing routine tonsillectomies at the Karolinska University Hospital Huddinge and Capio Ear, Nose and Throat Clinic in Stockholm, Sweden (**Paper I**). Patients visiting the specialized Emergency Department and the Infectious Diseases ward at Karolinska University Hospital in Solna (Sweden) were sampled for blood, nasal swabs and nasopharyngeal aspirates (NPA) (**Paper II**). Matched convalescent samples were collected from patients who returned for repeat sampling at least 4 weeks after acute phase sampling.

Bronchoscopies were performed on uninfected controls (UCs) and on HFRS patients to obtain endobronchial biopsies (EBB) and bronchoalveolar lavage (BAL) samples. HFRS patients were hospitalized at the University Hospital of Umeå, Umeå (Sweden) between 2008-2011 and underwent bronchoscopies on the earliest possible day during acute disease at which they could withstand the procedure and thrombocyte counts were higher than 100 x 10^9/L. Venous blood was also collected (CPTs, BD) from UCs and longitudinally from HFRS patients (**Paper III**). Venous blood samples (EDTA-containing vacutainers, BD) were collected longitudinally from

HFRS patients hospitalized at the Tampere University Hospital, Tampere (Finland) between 2002-2007 during acute disease and convalescence (**Paper IV**).

PROCESSING OF SAMPLES

Venous blood was centrifuged at 800g/10 min/room temperature (RT) to separate plasma (frozen at –20°C). The cellular fraction of whole blood and buffy coats were reconstituted/diluted 1:1 in sterile PBS. Density gradient centrifugation was performed using Ficoll-Hypaque Plus (GE Healthcare) at 900g/25 min/RT (without brake) to obtain peripheral blood mononuclear cells (PBMCs). PBMCs were either used fresh or cryo-preserved in FBS + 10% DMSO at –80°C. Tonsil tissue was disrupted mechanically with forceps and scissors, in R10 medium (RPMI 1640 medium + 10% FCS + 5 mM L-glutamine + 100 U/ml penicillin and streptomycin each). Sequential filtration was performed through 100μm and 70μm nylon cell strainers (Saveen & Werner) to obtain a single-cell suspension. Nasal swabs (Sigma Virocult®) were centrifuged and frozen in RNAlater (Thermo Fisher). NPA samples were filtered (70μm cell strainer) and centrifuged at 400g/5 min/RT. Supernatants were frozen at –20°C and NPA cells were washed with sterile PBS. BAL was obtained following 3 washes with 60mL saline solution (kept on ice), filtered through a 100 μm nylon filter and centrifuged at 400g/15 min/4°C to obtain BAL cells. EBB specimens were washed in dithiothreitol (DTT), fixed in acetone and embedded in glycol methacrylate (GMA) resin (Polyscience) for sectioning and immunohistochemistry.

ISOLATION OF DCs AND MONOCYTES

PDCs and CD1c+ MDCs were enriched from PBMCs and tonsil mononuclear cells (TMCs) using the Diamond Plasmacytoid Dendritic Cell Isolation Kit II and CD1c (BDCA-1)+ Dendritic Cell Isolation Kit (both Miltenyi Biotec). Enriched monocytes were obtained from buffy coats using the RosetteSep monocyte enrichment kit (StemCell Technologies). CD1c+ MDCs and CD16+ monocytes were isolated from enriched monocytes with CD1c+ Dendritic Cell Isolation Kit (Miltenyi Biotec) or CD16-conjugated microbeads (Miltenyi Biotec). MDDCs were generated also from enriched monocytes, by culturing them (0.5×10^6 cells per mL of R10) supplemented with recombinant human IL-4 (40 ng/mL) and GM-CSF (40 ng/mL) (Peprotech) for six days. PDCs in culture were supplemented with 1ng/mL IL-3 and CD1c+ MDCs, with 2ng/mL GM-CSF.

ENDOTHELIAL CELL CULTURE

Human primary dermal blood microvascular endothelial cells (BECs) were

maintained in culture in endothelial basal medium (EBM-2) and SingleQuots™ Kit (Lonza) and used at passages 7 to 10.

IN VITRO STIMULATION AND EXPOSURE TO VIRUS

In **Paper I**, PDCs were exposed to IAV (X-31, from Influenza A/Aichi/2/68; H3N2) *in vitro* at MOIs of 0.6 to 6.0 or CpG (ODN 2395; InvivoGen) at 1ng/mL; 3M-019 (7/8L; gift from Dr. R. Seder, National Institutes of Health). In **Paper II**, PBMCs were exposed to 1μg/mL 3M-019 (7/8L; Invivogen) for 2h to measure TNFα secretion. In **Paper III**, CD1c+ DCs and monocytes were exposed to PUUV strains Kazan or HTNV strain 76-118 (propagated in Vero E6 cells; ATCC) *in vitro* at an MOI of 0.1 to 7.5 for 2h followed by wash, and then incubated for up to 60h. Monocytes and CD1c+ MDCs (40h post-infection) were adhered on Alcian blue-coated coverslips for immunofluorescence analysis. In **Paper IV**, PBMCs were exposed to 1μg/mL 3M-019 (7/8L; Invivogen) for 3h to measure cytokine production. Brefeldin A (BFA, Sigma-Aldrich; 10μg/mL) was added after 30min. Enriched CD16+ and CD16– monocytes were exposed to PUUV strains Suonenjoki (propagated on MyGlaRec.B cells; EVAg) and Kazan (propagated on Vero E6 cells; ATCC) *in vitro* at an MOI of 1 for 1h followed by wash and incubation for 20h. BECs were infected with PUUV at an MOI of 10 for 1hr followed by wash and incubation for 72h; or treated with 50 ng/ml TNFα (R&D systems), for 72h. Monocytes were added to BECs at a 5:2 cell to cell ratio.

FLOW CYTOMETRY

Cells were first stained with LIVE/DEAD Fixable Dead Cell kit (Invitrogen), then blocked with FcR-blocking reagent (Miltenyi Biotec). Surfaced staining was performed with respective panels of antibodies conjugated to fluorescent dyes. Cells were analyzed after fixation with 1% PFA on the BD LSRII or BD LSRFortessa (BD). For intracellular staining to detect viral nucleoprotein (NP) or cytokines, cells were fixed and permeabilized using a staining buffer solution (eBioscience) and antibodies against IAV NP, TNFα or IL-6. TNFα release from PBMCs over 2h of stimulation with 3M-019 (7/8L) was assessed using the TNFα Secretion Assay-Detection Kit (PE) (Miltenyi Biotec) incorporated into the surface staining protocol.

MICROSCOPY

GMA-embedded EBB sections (2μm) were stained with respective mouse primary antibodies and rabbit anti-mouse biotinylated secondary antibodies. Sections were analyzed using a digital scanner (NanoZoomer-XR; HAMAMATSU). PUUV- exposed monocytes and CD1c+ MDCs were stained with human anti-PUUV serum followed by an anti-human IgG secondary antibody conjugated to Alexa Fluor 488. Confocal

imaging was performed on a Zeiss LSM700 (10x objective) and visualized using FIJI ImageJ software (NIH).

NUCELIC ACID AND PROTEIN ANALYSES

Total RNA from tonsil and blood PDCs was extracted using RNeasy kit (QIAGEN) and cDNA was reverse-transcribed using a kit (Applied Biosystems). A preconfigured TaqMan low-density array (48 genes) was used to detect gene expression relative to housekeeping genes by qRT-PCR. Lysates of tonsil and blood mononuclear cells and PDCs (± exposure to IAV for 12h) were collected in RIPA buffer (Sigma-Aldrich) and analyzed by standard western blotting on a PVDF membrane. MxA expression was detected using a primary antibody (Genentech) and HRP-conjugated secondary antibody (Thermo Fisher Scientific).

6 RESULTS AND DISCUSSION

As discussed in the introduction, MNPs from different anatomical compartments have distinctive features and different functional capabilities. **Papers I and II** were designed to identify the differences in the distribution and function of human MNPs from blood and the upper respiratory tract, in the context of Influenza A virus infection. **Paper I** allowed comparison of IFN responses of blood and tissue-resident DCs to IAV exposure *in vitro.* Paper I helped answer fundamental questions about PDC biology, their susceptibility to IAV infection and their ability to produce IFNα. The findings in Paper I complemented the hypothesis and findings from **Paper II**, where we sought to assess MNP recruitment to the nasopharynx during ongoing human influenza infection with seasonal IAV strains. Paper II allowed detailed characterization of local and systemic MNP distribution, maturation and function, in conjunction with inflammation. Paper II, however, provided only a snapshot view at the immune system and we were also limited by nasopharyngeal cell numbers to perform further functional analyses. **Paper III** complemented Papers I and II by extending the course of disease with longitudinal sampling of patients, albeit in a different disease model, of HFRS during Puumala virus infections. Additionally, bronchoscopy investigations of the patients and controls augmented the findings observed in blood, and demonstrated the redistribution of MNPs in blood and airways during acute viral infection. **Paper IV** provided us the opportunity to assess if different Puumala viruses induced differential patterns of MNP redistribution during acute HFRS. Additionally, we were able to evaluate if circulating MNP were functionally impaired during the viremic phase of infection. Taken together, some of the results I will discuss in the following sections highlight the diverse and unique features of human MNP behavior during acute viral infections. The unabridged findings can be found in the corresponding original **Papers I-IV**.

6.1 DIFFERENTIAL IFNα RESPONSES OF HUMAN TONSIL AND BLOOD PDCS TO I*N VITRO* IAV EXPOSURE (PAPER I)

PDCs have been considered refractory to virus infection, a feature attributed to their rapid and potent IFN-producing abilities [277, 292] and their constitutive expression of the interferon-stimulated gene (ISG), MxA [279]. In particular, PDCs have demonstrated resistance to IAV infection under experimental conditions, whereas CD1c+ MDCs are readily susceptible [14]. As PDCs are rare in circulation, and rarer still in non-lymphoid tissues at steady state [293], detailed comparisons between blood and tissue-resident human PDCs have been limited. In **Paper I**, we hypothesized that human PDCs from blood and mucosal tissues differ in functionality. We tested this hypothesis by obtaining PDCs from human tonsils,

lymphoid organs rich in PDCs [215], and resident in the upper respiratory tract- the initial site of IAV infection.

We first obtained CD123+ PDCs with high purity (>95%) from both blood (buffy coats) and tonsils by depleting non-PDCs with microbeads. As expected, tonsils yielded significantly more PDCs than blood (**Figure 11A**) [230]. Using a custom-designed low-density gene array, we assessed differential gene expression between enriched blood and tonsil PDCs. We observed no striking differences in the genes expressed between the PDCs from both tissues, only differences in level of expression. We detected comparatively higher expression of IRF7 and IFNB in tonsil PDCs, and higher expression of TLR7, TLR9, IRF3, MYD88, IFNAR1, IFNA1, IL-6 and TNF in blood PDCs. Overall, we observed higher expression of IFN-signaling associated genes [279, 294, 295] in blood PDCs than in tonsil PDCs.

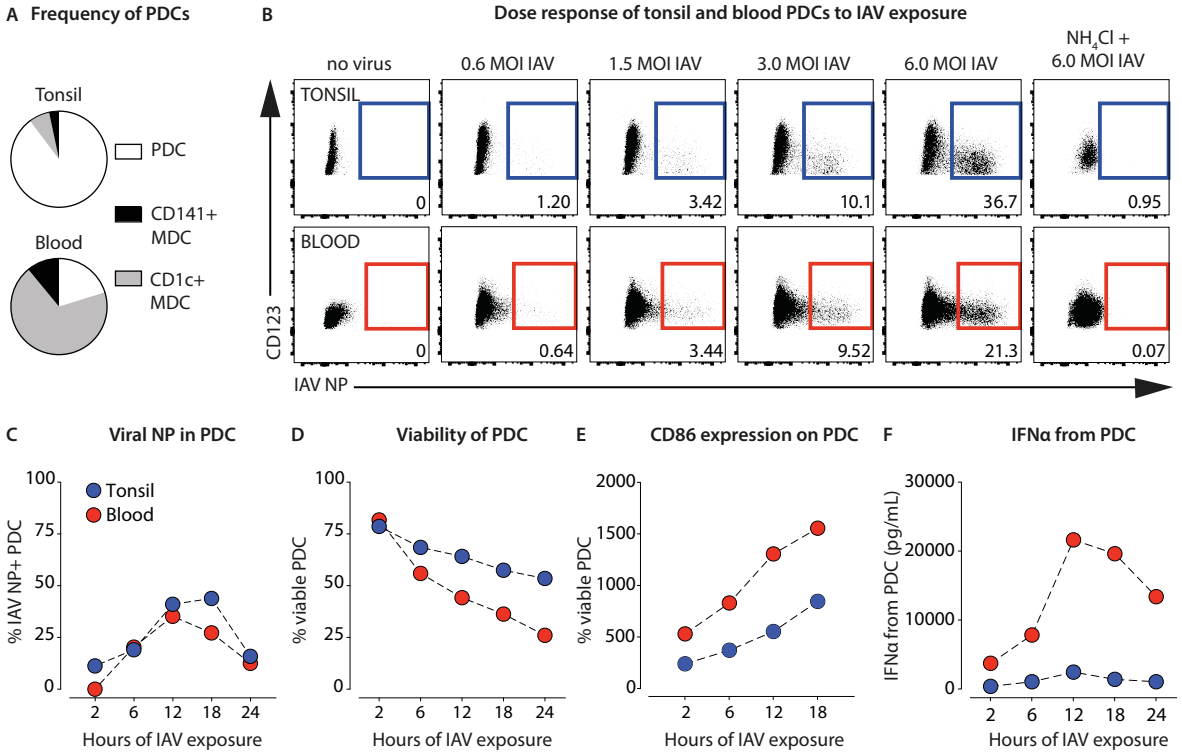

Figure. 11. Human tonsil and blood PDCs are both susceptible to IAV infection in vitro, but tonsil PDCs display attenuated maturation and IFNα responses. (A) PDCs are the most abundant DC subset in the tonsils whereas in blood, CD1c+ MDCs are more frequent. (B) Upon exposure to increasing MOI of IAV, both tonsil and blood PDCs are susceptible to infection at MOI<3.0 (flow plots from representative donors). (C) At an MOI of 6.0, tonsil (n=27) and blood (n=18) PDCs display peak median levels of IAV nucleoprotein intracellularly at 12-18h and 12h of *in vitro* exposure respectively. Blood PDCs, however, had comparably (D) lower median viability over time and (E) higher median expression of CD86 than tonsil PDCs. (F) Importantly, blood PDCs (n=12) produced at least 10-fold higher median IFNα at peak infection (12h) as compared to tonsil PDCs (n=9), despite similar pattern of IFNα induction over time.

To test if the steady-state antiviral resistance of PDCs could be overcome by a high virus dose, we exposed PDCs to increasing amounts of IAV for 12h and found viral

nucleoprotein (NP) in both tonsil and blood PDCs at MOIs up to 6.0 (without complete loss of PDC viability) (**Figure 11B**). We studied the kinetics of viral replication in PDCs over 24h, quantifying the number of viral NP+ cells using flow cytometry. Peak NP+ PDC frequencies were observed at 12h in blood PDCs and 12-18h in tonsil PDCs, after which we observed a decline in NP+ PDCs (**Figure 11C**). The viability of IAV-exposed PDCs continued to decrease over time as viral replication continued [296]. Strikingly, blood PDCs were less permissive to IAV replication than tonsil PDCs, and also succumbed to cytopathic effects faster (**Figure 11D**). Blood PDCs also matured more than tonsil PDCs, both in the absence or presence of IAV, upregulating their level of CD86 expression (**Figure 11E**).

The most striking finding however, was observed in the IFNα responses. Blood PDCs demonstrated potent secretion of IFNα, and significantly higher amounts than tonsil PDCs, with approximately 10 times higher IFNα detected at 12h of IAV exposure (peak virus NP) (**Figure 11F**). Blood PDCs also expressed higher quantities of the ISG MxA than tonsil PDCs upon exposure to IAV. The diminished IFNα secretion and subsequent antiviral effector expression we observed in tonsil PDCs may explain their higher permissiveness to viral replication than blood PDCs [281, 297]. Tonsil PDCs also displayed dampened IFNα, IL-6 and TNFα responses to CpG (TLR9 agonist) or 3M-019 (TLR7/8 agonist) stimulation as compared to blood PDCs leading us to propose that tonsil PDCs exhibit overall muted responses to IAV exposure or TLR agonist stimulation as compared to blood PDCs.

The findings in **Paper I**, highlighted the importance of studying immune cells like DCs from tissues in addition to blood, as we discovered significant functional differences between PDCs from different tissues. In the context of IAV infection, where the virus initially infects the epithelium in the upper respiratory tract, replicates *in situ*, limited *in vitro* analysis with primary DCs obtained from blood or MDDCs provides a partial, and sometimes inaccurate, understanding of DC function. Therefore, experimental setups like in **Paper I** are essential for critical *ex vivo* evaluation of DC responses to IAV exposure. A limitation of the present study is that tonsils and blood originated from different individuals. A comparison of PDC responses from different tissues in the same individual would enhance the strength of our findings.

It must be noted though, that locally (in the upper respiratory tract), tonsil PDCs could compensate for individually dampened responses by sheer excess of numbers due to relative abundance in the tonsils. On the other hand, perhaps the requirement for (and suitability of) a potent inflammatory response is lower in the upper respiratory tract. The location of the tonsils constantly exposes them to inhaled particles, antigens and pathogens compelling a muted inflammatory response in the tonsils [298]. Furthermore, blood PDCs, while exhibiting potent IFNα responses, perish sooner from the cytopathic effects of IAV. Tonsil PDCs may have a small

survival advantage over blood PDCs resulting in prolonged IFNα secretion. Due to the *ex vivo* nature of the study, we were unable to assess the contribution of DC mobilization to the upper respiratory tract during human IAV infection, as has been reported previously [196, 197].

6.2 RECRUITMENT OF MONOCYTES AND DCs TO THE NASOPHARYNX DURING HUMAN IAV INFECTION (PAPER II)

The recruitment of monocytes and DCs to the human respiratory tract during severe IAV infection has been reported previously [197, 198]. However, a majority of the clinical studies have been performed on patients with serve influenza disease who require hospitalization. For example, key studies on the 2009 pandemic H1N1 IAV patients have been instrumental in identifying correlates of disease severity in pandemic influenza. Nasal CCL7, and IL-10 [198]; TNFα-producing M1-like proinflammatory monocytes [254], plasma IL-1β, IL-6, CXCL-8, IL-8, CCL2 and sTNFR1 [259, 299]; and suppressed Th1/Th17-related cytokines CXCL10 (IP-10), CXCL9 and IL-17A [300] were all reported to be predictors of severe influenza disease. Similar studies are required for seasonal IAV infections, which present with a broad range of severity- ranging from asymptomatic infection to respiratory disease to, in some instances, death (**Figure 1**). To address this void in the field, in **Paper II**, we characterized blood and nasopharyngeal MNPs in patients seeking healthcare with seasonal IAV infections during acute and convalescent disease. Investigations of nasopharyngeal monocyte and DC composition have been uncommon due to the physical challenges of sampling, high risk of contamination with cells from blood vessels and the rarity of monocytes and DCs. Some previous studies on nasal cells using flow cytometry were also limited technically, in the ability to detect multiple markers at the same time [196-198]. Using an extended flow cytometry panel, we were able to characterize the 3 monocyte subsets and the 3 major DC subsets from both blood and the nasopharynx in their phenotype, maturation and migration marker expression.

During 3 consecutive influenza seasons of 2016-2018, we included adult patients seeking healthcare with symptoms of influenza-like illness, who presented with fever and one or more of the following symptoms: cough, nasal congestion, headache or muscle ache. We excluded patients with immunodeficiencies and patients who were taking antibiotics, immunomodulatory or anti-inflammatory medication at the time of inclusion. Of the 84 patients included in the study, 40 patients were PCR+ for IAV infection. The IAV– patients, a heterogeneous group with symptoms of IAV infection but really had IBV, RSV or other viral or bacterial infections, were also assayed similarly but excluded from analysis. Patients were asked to return for follow-up sampling at least 4 weeks later, ascertained to be IAV– by PCR, to be sampled again. 16 healthy controls (HCs) were also included, although the broad age range of the IAV patients (20-98) made it difficult to find exactly age-matched healthy

individuals. We analyzed blood and nasopharyngeal aspirates (NPA) from each study participant (**Figure 12A**). We also collected detailed patient histories and obtained blood chemistry results.

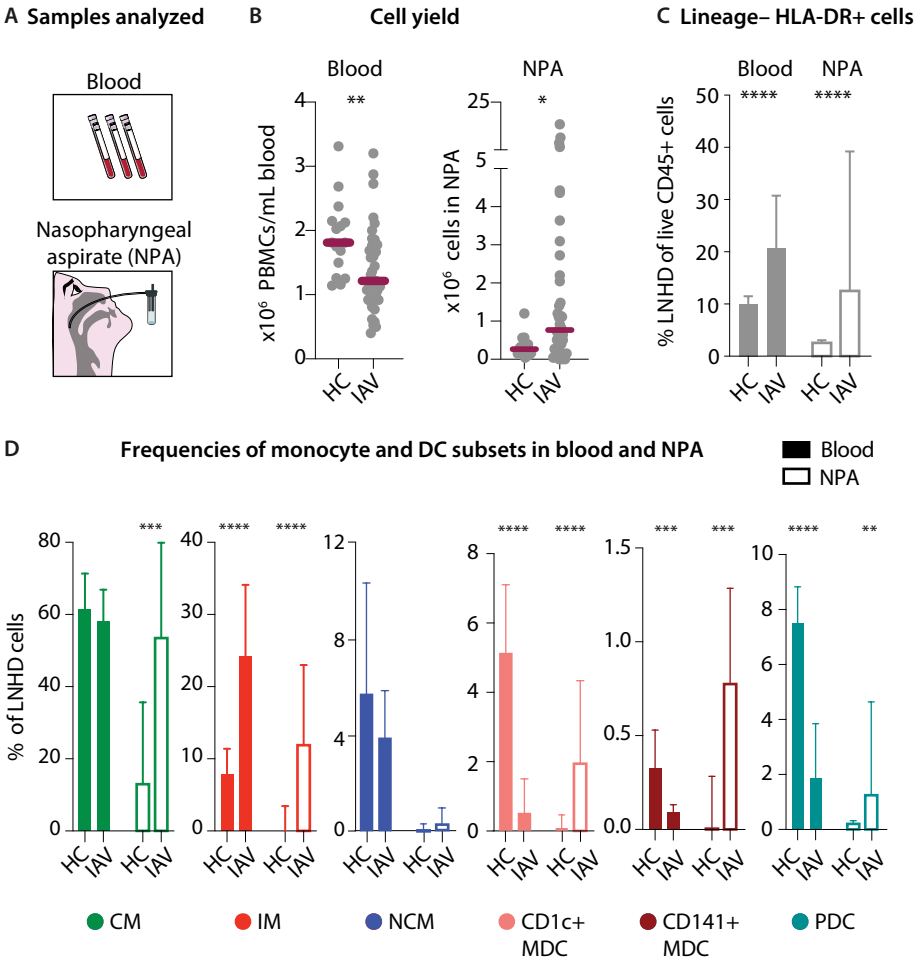

Figure 12. Acute seasonal IAV infection recruits monocytes and DCs to the human nasopharynx. We assessed immune cells in (A) blood and nasopharyngeal aspirates of patients using flow cytometry. (B) IAV patients (n=40) had fewer PBMCs in blood, and a significant cellular influx to the nasopharynx during acute IAV infection, as compared to HCs (n=16). (C) In both, blood and the NPA the relative frequency of **l**ineage (CD3/CD19/CD20/CD56/CD66) **n**egative **H**LA-**D**R (**LNHD**) cells was increased in IAV patients (n=22) as compared to HCs (n=12). (D) In the LNHD compartment in blood, IMs were significantly increased, and all the DC subsets were reduced in frequency. In the nasopharynx, IAV infection was accompanied by infiltration of CMs, IMs, CD1c+ MDCs, CD141+ MDCs and PDCs.

During IAV infection, patients had cell infiltration to the nasopharynx, as compared to HC (**Figure 12B**). By flow cytometry, we were able to demonstrate that IAV patients have increased frequencies of **l**ineage **n**egative **H**LA-**D**R-expressing (**LNHD**) cells- the compartment where monocytes and DCs are typically identified- in both blood and in the nasopharynx (**Figure 12C**). In the monocyte compartment, CD14+CD16– **CMs** remained unchanged in blood, but were significantly increased in the nasopharynx, accounting for the bulk of the immune cell infiltration seen in this compartment during IAV infection (**Figure 12D)**. Monocytes and DCs in the nasopharynx of patients were also more mature than their counterparts in blood,

expressing more HLA-DR and CD86; and also in comparison to HCs. Additionally, CD86 expression on CMs correlated with viral load (inverse correlation with Ct value)- i.e., higher viral loads appeared to drive DC and monocyte maturation at the site of IAV infection.

Additionally, CD14+CD16+ **IMs** were significantly elevated in frequency in blood and the nasopharynx (**Figure 12D**). Similar observations have been reported before, for other virus infections and in sepsis [192, 193]. Interestingly, in patients with the 2009 pandemic H1N1 IAV infection, two geographically distinct cohorts reported strikingly increased frequencies of IMs in blood of patients [198, 254]. Only Oshansky *et al.* have previously reported increased CMs and IMs in nasal lavage of pandemic IAV patients. We were able to confirm that seasonal IAV infections also appear to elicit a similar pattern of alteration in monocyte phenotype. Additionally, we also observed that age was correlated positively with IMs in the nasopharynx and inversely correlated with IMs in blood (also reported in [198]). IMs in blood and the nasopharynx of IAV patients were more mature than in HCs. Blood IMs also upregulated CCR2 expression, and their increased frequency correlated with levels of plasma CCL2 (or MCP-1, monocyte chemoattractant protein 1). Previous studies comparing patients with seasonal or pandemic H1N1 IAV infections showed correlation of CCL2 with complications and pneumonia in hospitalized patients [259, 300]. Interestingly, in our study cohort, age had an inverse correlation with IM frequencies in blood, and possibly with plasma CCL2. As we assayed plasma CCL2 in only a subset of our patients, we lacked the power to test the effects of age directly on CCL2 levels. As a potent inducer of monocyte migration, CCL2 gradients in blood versus the nasopharynx can dictate severity of inflammation [301]. In murine models, a PPARγ agonist-mediated suppression of CCL2 secretion, and therefore of TNFα/iNOS-producing DCs was shown to ameliorate disease severity [261]. The IM-CCR2-CCL2-TNFα axis may be crucial to reducing influenza severity and merits further investigation.

We detected the 3 major **DC** subsets- **CD141+ MDCs** (cDC1), **CD1c+ MDCs** (cDC2) and **PDCs** in blood; and in a subset of HCs also in the nasopharynx. Often, NPA samples from HCs yielded few to no DCs, as we expected. In IAV patients, all 3 DC subsets were found in strikingly reduced frequencies in blood, and in comparably increased frequencies in the nasopharynx in IAV patients (similar to previous reports in pediatric RSV and IAV infections [196, 197]). We also observed an inverse correlation between age and the frequency of CD1c+ MDCs- in contrast to the positive correlation seen with IM frequencies. Nasopharyngeal DCs in the patients were also more mature as compared to DCs in blood, with MDC maturation correlating with CM maturation (which in turn correlated with viral load).

The lowest CM frequencies were observed in blood at day 1 but in the nasopharynx, peak CM frequencies were seen later (day 3) than in blood. Overall, CM frequencies

were seen to fluctuate over disease course, leading us to speculate if CMs were being replenished from the bone marrow as has been postulated before [149]. CMs could either be recruited to the nasopharynx, or be differentiating into IMs in circulation, or as we suspect- both. Peak IM frequencies were also found earlier in blood (day 1) than in the nasopharynx (day 3-4) during IAV infection. In the nasopharynx, peak frequencies of the different DC subsets were observed at different days of symptoms, suggesting differential kinetics of their recruitment to, or differentiation in, the nasopharynx. It is currently unclear if in addition to CMs, IMs and DCs are also actively recruited to the nasopharynx, or the cells we identified there were a consequence of local differentiation from CMs. In a human experimental endotoxemia model, LPS injection induced loss of all 3 monocyte subsets from circulation within 2 hours [149]. Whether IAV infection is sufficient to induce similar recruitment of all monocytes (and DCs) to the nasopharynx, remains to be tested with human experimental challenge models with IAV [74, 94], or using the live-attenuated influenza vaccine (LAIV) to mimic a human infection model.

We also observed increased levels of the cytokines **TNFα** (**Figure 13A**), IL-6 and IFNα in both plasma and the nasopharynx. In both compartments, cytokine levels correlated with CM frequencies. Supporting this, we found that it was older patients, who had more CMs in blood than IMs, that had higher plasma TNFα levels. Since TNFα was elevated locally and systemically during IAV infection, we sought to identify its cellular source. Due to limited cell yields from the nasopharynx, we had to limit our functional studies on the MNPs from blood in patients and HCs. We used a TNFα-secretion assay which immobilizes any TNFα released by a cell on the surface of the same cell, enabling simultaneous detection of the cytokine secreted and the phenotype of the cell via flow cytometry (**Figure 13B**). In IAV patients, unstimulated monocytes and DCs release TNFα (mostly from CMs and IMs) as compared to cells in HCs (**Figure 13C**). Upon further stimulation with a TLR7/8 agonist, all monocyte and DC subsets in patients and HCs produce TNFα, with stimulated cells from patients often exceeding the levels seen in HCs. Therefore, monocytes and DCs in blood actively contribute to systemic inflammation in a TNFα-mediated manner. In line with previous reports of TNFα-expressing monocytes contributing to severe disease independent of comorbidities [254], we showed that CMs and IMs contribute to seasonal influenza disease. Mature CMs, IMs and DCs are all present in the nasopharynx during infection in the same individuals. And we observe a correlation between plasma and nasal TNFα. We can extrapolate from our findings to propose that monocytes and DCs recruited to the site of infection, the human nasopharynx, contribute to TNFα-dependent local inflammation.

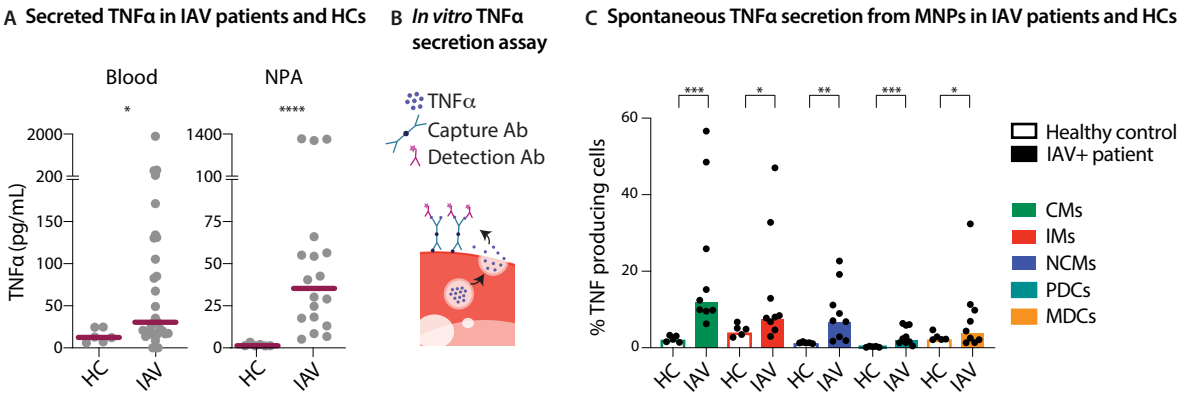

Figure 13. During IAV infection, monocytes produce TNFα. (A) IAV infection (n=31) is marked by high levels of TNFα in circulation (IAV: n=31) and locally, in the nasopharynx (IAV: n=18), as compared to HCs (n=12). (B) We measured spontaneous and TLR agonist-stimulated TNFα production by MNPs in circulation, using an *in vitro* TNFα secretion assay. (C) In IAV patients (n=9), all monocytes and DCs spontaneously secreted TNFα during acute infection, as compared to HCs (n=5). CMs and IMs produced the highest amount of TNFα among the MNPs.

Lastly, we sampled IAV patients during convalescence, to assess if the changes we observed in MNPs were normalized to reflect levels seen in HCs. Indeed, the frequencies of CD1c+ MDCs were increased in blood and reduced in the nasopharynx; and IMs were reduced in both compartments. With **Paper II**, we have shown that a broader understanding of underlying immune processes can be obtained by sampling the respiratory tract during infection in a less invasive manner. We also demonstrate, once again, the importance of studying MNPs at the site of infection in addition to MNPs from blood.

6.3 REDISTRIBUTION OF MONOCYTES AND DCs IN PERIPHERAL BLOOD AND AIRWAYS OF ACUTE PUUV-HFRS PATIENTS (PAPER III)

Hantaviruses are transmitted via inhalation similar to influenza viruses, but are quite different in their pathogenesis. In Sweden, the endemic strain of hantavirus, Puumala virus, causes hemorrhagic fever with renal syndrome (**HFRS**). The target of the virus is the vascular endothelium, which supports replication without the manifestation of cytopathic effects. HFRS is accompanied by exaggerated immune responses, typically mediated by NK cells and CD8 T cells. Monocytes and DCs in particular, may contribute to both orchestration of the adaptive responses, and also to the inflammation seen in HFRS, both of which contribute to vascular leakage. The *in situ* immunological events following the initial entry of the virus via the airways, and replication in vasculature of the airways are poorly understood. Although HFRS primarily causes renal dysfunction, increasingly, respiratory distress has been reported in these patients [122, 284] as hantaviruses replicate in the lower airways. However, the thrombocytopenia associated with acute HFRS and the risk of

aerosolizing cell-free virus restrict airway sampling in general and dismiss longitudinal sampling of the airways. Despite this limitation, in **Paper III**, we performed bronchoscopies (biopsies and lavage) on 17 patients with acute HFRS due to Puumala virus infection (as early as possible) and 12 age-matched uninfected controls (UCs); and obtained peripheral blood samples (**Figure 14A**).

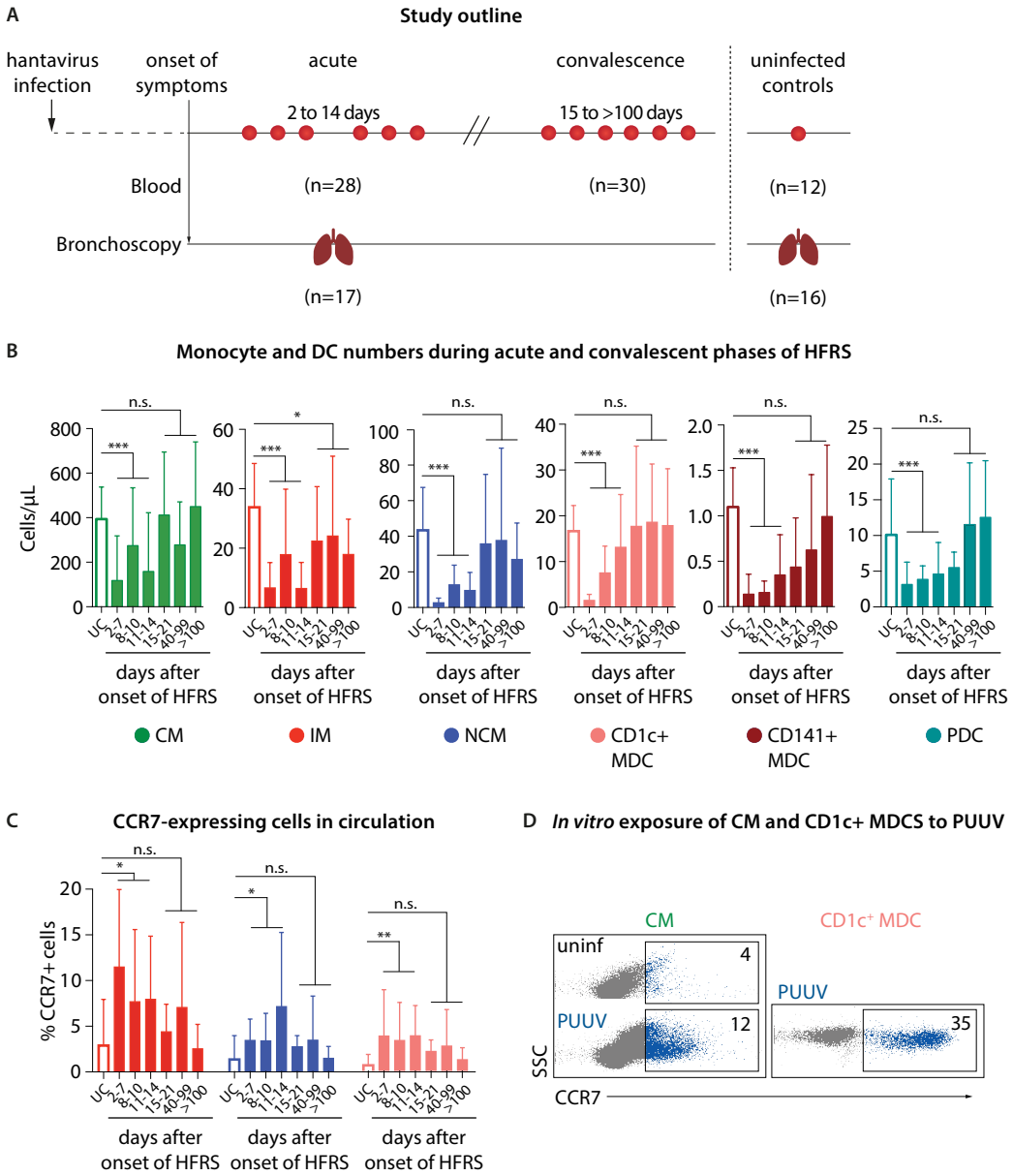

Figure 14. Acute HFRS causes redistribution of monocytes and DCs. (A) We obtained blood samples during acute (n=28) and convalescent (n=30) HFRS from 17 patients and 12 uninfected controls (UCs). Bronchoscopies were performed on all patients during acute disease and on 16 UCs. (B) During acute HFRS, peripheral blood frequencies of all monocyte and DC subsets were severely reduced and normalized to levels seen in UCs during convalescence. (C) IMs, NCMs and CD1c+ MDCs upregulated CCR7 in acute HFRS, suggesting migratory behavior. (D) When exposed to PUUV *in vitro*, CMs (n=4) and CD1c+ MDCs (n=6) also upregulated CCR7 (flow plots from representative donors).

Immunohistochemistry revealed significant infiltration of CD8+ T cells and HLA-DR+ cells into the airways of patients during acute HFRS as compared to UCs, and was confirmed by flow cytometry on BAL. CD8+ T cell infiltration to bronchial tissues in

HFRS associated with CD123+ cell infiltration. However, the effects of HFRS are largely systemic and viremia during HFRS was consistently present in blood over BAL. Therefore, we characterized monocytes and DCs in blood using flow cytometry, in an attempt to understand the MNP responses to the virus and/or inflammatory cytokines in circulation; and how MNPs may contribute to CD8+ T cell infiltration and activation.

During acute HFRS, **CMs**, **IMs** and **NCMs** were all reduced in numbers in circulation, as compared to UCs (**Figure 14B**). During convalescence, when viremia was reduced, the numbers of monocytes returned to steady-state levels. Similarly, numbers of **CD1c+ MDCs**, **CD141+ MDCs** and **PDCs** were all strikingly reduced in circulation during viremic HFRS and normalized during convalescence. We next examined for evidence of monocyte and DC migration to peripheral tissues [302, 303] or to the lymph nodes [304] by assessing CCR7 expression. Notably, the IMs, NCMs, CD1c+ MDCs and PDCs remaining in blood during acute disease all displayed increased CCR7 expression as compared to UCs or during convalescence (**Figure 14C**). Taken together, we found that acute HFRS was characterized by a marked depletion of monocytes and DCs, and cells remaining in blood, were both mature, and showed indications of migratory signaling.

Additionally, we also performed *in vitro* experiments where we exposed CMs and CD1c+ MDCs to PUUV. PUUV did not induce any cytopathic effects in the cells, but induced changes in chemokine receptor expression (**Figure 14D**). This observation supports our idea that loss of MNPs during viremic infection may be due to migration as opposed to death. We also noted that CCR2 expression was reduced but CCR4, CCR6 and especially CCR7 expression, were all increased on CMs and CD1c+ MDCs, suggesting the CCR7 expression we observed on MNPs in patients is a possible consequence of PUUV exposure.

If MNPs indeed migrate to the airways during HFRS, their presence may explain the increased CD8+ T cells in the airways and increased respiratory symptoms seen in HFRS [118, 122, 284]. Further investigations are required to understand the dynamics of MNP redistribution and activation during HFRS and their individual contributions to inflammation.

6.4 PRONOUNCED DEPLETION OF NONCLASSICAL MONOCYTES DURING SEVERE PUUMALA VIRUS HFRS (PAPER IV)

To expand on our findings in **Paper III**, in **Paper IV**, we enquired if different Puumala virus strains associated with somewhat different clinical phenotypes, elicit distinct patterns of MNP mobilization and/or redistribution. We also sought to assess the effects of viremia in HFRS on the functions of MNPs. Lastly we tried to identify innate immune determinants of HFRS severity.

We studied a cohort of 23 HFRS patients from Tampere, Finland who presented with renal symptoms (unlike the Swedish cohort in **Paper III**). We obtained acute and convalescent phase peripheral blood samples from all patients and 9 UCs. Acute disease in patients was marked by thrombocytopenia, and elevated CRP and creatinine, which subsided during convalescence. We also calculated an adapted severity score (**a-SOFA**; based on thrombocyte counts, creatinine levels and mean arterial pressure readings) and stratified patients into low (a-SOFA<5) or high (a-SOFA≥5) severity groups. Patients with higher severity also had higher total leukocytes during acute HFRS.

Monocyte subsets were identified using flow cytometry, and the most striking observation involved the **NCMs**, which were significantly depleted during acute disease as compared to UCs (**Figure 15A**). Patients with severe disease were particularly depleted of NCMs during acute disease, and the NCM frequencies remained below median levels seen in UCs even during late convalescence (day 360) (**Figure 15B**). Meanwhile **CMs** (n.s.) and **IMs** (significant) were elevated during acute disease, indicating restructuring of the monocyte compartment during acute HFRS. No major changes were observed in the **DC** compartment. Overall, the pattern of monocyte distribution in this cohort was different from what we observed in **Paper III**. The variation we report may be attributed to the different virus strains in circulation in Northern Sweden and Eastern Finland [101, 305] as patients in the current cohort exhibited no obvious pulmonary complications. Detailed phylogenetic analysis of the two viruses would provide clarification. An alternative hypothesis is that the current cohort does include samples from patients prior to 5 days with symptoms. And lastly, in both instances, the days with symptoms are self-reported by the patients and may introduce additional variation by altering the physiological window being analyzed. The viral loads in both cohorts, although, were comparable (~10^4 to 10^6 viral copies/mL of plasma).

Acute HFRS was also marked by IMs and NCMs upregulating surface expression of HLA-DR, CCR2, and CCR7, as compared to UCs, which normalized during convalescence. CCR2 expression on IMs was particularly elevated in patients with severe disease who we suspected may have higher systemic inflammation (behaving similar to the **IMs from IAV+ patients** in **Paper II**). However, cytokine responses were not comparable between both groups of patients, with only mildly elevated plasma cytokine levels seen in the HFRS cohort. Elevated cytokine levels in plasma, though, correlated well with elevated creatinine levels and thrombocytopenia.

Similar to our observations in **Paper III**, all monocyte subsets had increased CCR7 expression during acute HFRS, suggestive of signaling towards peripheral migration. Strikingly, the patients with less severe disease had higher CCR7 expression on IMs and NCMs than patients with severe disease (**Figure 15C**). Efficient CCR7 signaling

may induce sufficient recruitment of monocytes to the lymph nodes in these patients, reducing severity of their disease.

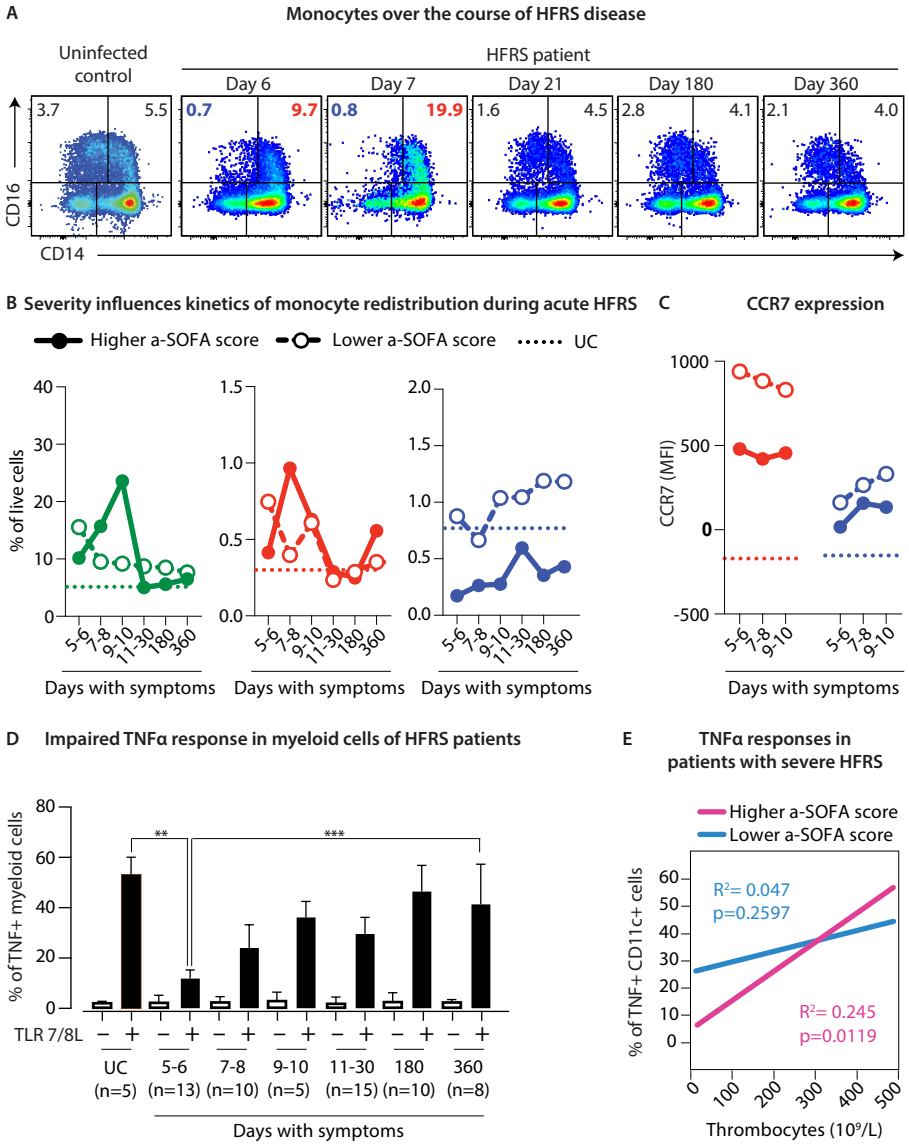

Figure 15. Severe HFRS is accompanied by a distinct loss of nonclassical monocytes and impaired TNFα secretion. (A) During acute HFRS, NCMs were severely depleted in blood as compared to an uninfected control (UC), reappearing during convalescence (flow plots from representative donors). (B) Patients with severe HFRS (higher a-SOFA score) displayed dramatically low NCM frequencies during acute disease; with peak CM and IM frequencies observed later than in patients with lower a-SOFA scores. (C) IMs and NCMs in patients with severe disease upregulated CCR7 to a lower extent suggestive of delayed recruitment of CMs and IMs to the periphery during severe disease. (D) CD11c+ myeloid cells during acute HFRS were impaired in their ability to respond to TLR7/8 agonist stimulation and secrete TNFα as compared to UCs. (E) The extent of impairment of TNFα response correlated with level of thrombocytopenia (in patients with severe disease).

Conversely, severe disease may be aided by weaker recruitment of monocytes. This hypothesis also supports our observation of peak CM and IM frequencies later in severe patients as compared to patients with less severe disease (**Figure 15B**). The myeloid cell compartment (composed primarily of monocyte subsets) in HFRS patients was impaired in its ability to respond to further TLR stimulation (quantified in

terms of TNFα secretion) in the earliest period of acute HFRS, suggestive of exhaustion in this compartment (**Figure 15D**). Patients with the lowest thrombocyte counts (and severe disease) were also most impaired in TNFα release (**Figure 15E**). Assaying the responses of blood, however, only offers incomplete knowledge of inflammatory processes as we and others have demonstrated. Therefore, further testing is required to identify the fate of the monocytes after (likely) exposure to virus, and address their exact contribution to HFRS. For example, acute kidney injury and renal dysfunction are hallmarks of HFRS but are typically assessed indirectly [138]. A recent study showed correlation between excreted IL-6 (in urine) and proteinuria in HFRS, suggesting local proinflammatory cytokine production- perhaps by monocytes or macrophages recruited to the kidneys [137]. So, the patients with severe thrombocytopenia, and impaired TNFα peripheral blood responses, may in fact have cytokinemia in the kidneys, if monocytes were present in increased numbers in the kidneys during acute HFRS.

During acute HFRS, IMs retained within vasculature, likely differentiate into NCMs [149], replenishing the depleted NCM pool. The CD16+ monocytes (IMs and NCMs), in addition to CCR2 and CCR7, also upregulated their expression of the endothelial adhesion marker CD62L (or L-selectin), which helps leukocytes tether and “roll over” endothelial cells. The underlying reason for, and the ultimate fate of the seemingly depleted NCMs is currently unknown. *In vitro*, we tested if CD16+ monocytes (IMs + NCMs) and CD16– monocytes (CMs) demonstrate differential adhesion and migration behavior upon exposure to cell-free hantavirus or hantavirus-infected endothelial cells. Recapitulating the responses seen in patients, *ex vivo* CD16+ and not CD16–monocytes upregulated CD62L expression. CCR7 expression was only mildly elevated, and only when cells were exposed to virus-infected endothelial cells. Preliminary results suggest that CCR7 upregulation may require both cell-free and endothelial cell-associated virus (as in human infection), and additional TLR signaling or soluble factors.

Typically patrolling the vasculature, NCMs are retained in capillaries for ~9 minutes at steady-state [149, 268]. In murine models, NCMs respond to inflammation in the endothelium by recruiting neutrophils to kidneys in a TLR7-dependent manner. The neutrophils proceed to cause endothelial cell necrosis and the monocytes remove cellular debris [201, 268, 269]. While this mechanism has not been investigated in humans, it is likely that NCMs behave similarly, offering an explanation correlating the loss of NCMs and the renal dysfunction in HFRS. Perhaps, in patients with severe disease, more NCMs are lost from circulation due to increased retention in the renal capillaries. Whether NCMs support or mitigate inflammation and subsequent vascular leakage during HFRS, remains to be understood with further investigation.

7 CONCLUSIONS

The constituent studies of this thesis were aimed at understanding how monocyte and DC distribution and function vary during human influenza A virus (IAV) and hantavirus infections in order to decipher their role in viral pathogenesis. Monocytes and DCs are critical orchestrators of the innate immune response to virus infection. *In vitro* studies and animal models have significantly improved our understanding of their ontogeny, biology and function, both at steady state and in disease models. Still, aspects of their function remain unknown, especially in the context of human disease. Furthermore, the anatomical dependency of MNP function requires comparative investigations of MNPs in blood and tissues, also in humans. With viruses of global health significance, the shortage of effective therapeutic interventions urges us to find alternative strategies to treat disease. Modulating the immune response to fight infections, especially in diseases with immune-mediated mechanisms of pathogenesis, require extensive knowledge and understanding of the host immune response. Characterizing the dynamics of innate responses to IAV and hantavirus infections, and searching for markers of disease severity will help uncover new targets for intervention. Once achieved, the discoveries made in a lab must be translated to the clinic and can eventually contribute to protecting human health.

In summary, the major findings from this thesis are as follows:

- Human DCs from distinct anatomical locations have different abilities to mediate antiviral protection (**Paper I**).

- During acute influenza infection, monocytes and DCs are recruited to the nasopharynx where they can promote inflammation (**Paper II**).

- During acute HFRS, circulating monocytes and DCs are activated and migrate from blood to the peripheral tissues and/or lymph nodes (**Paper III**).

- Severe HFRS causes pronounced loss of patrolling monocytes from peripheral circulation, as monocytes may be held within the blood vessels of the kidneys (**Paper IV**).

8 FUTURE DIRECTIONS

The findings in the current thesis have increased our understanding of monocytes and DCs, but also exposed many remaining questions that can be explored with future studies.

In **Paper I**, we established a novel system to study PDCs from the site of IAV infection and compare their responses to blood PDCs *ex vivo*. Human tonsillar tissue supports explant cultures and have been previously used to model HIV infection in mucosal tissues [306-308]. Tonsil explants can be used to study how IAV infection influences PDC behavior within the tissue architecture. We can assess if PDCs egress from the tonsils to the nasopharyngeal epithelium in response to IAV infection. Whether the attenuated IFNα responses are still protective to surrounding cells, or if tonsil PDCs promote recruitment of blood PDCs to the site of infection. Unlike *ex vivo* suspension cultures, explant systems support extended experimental periods, enabling us to map the kinetics of *in situ* PDC responses to IAV.

In **Paper II**, we capitalized on clinical collaborations, our existing expertise in flow cytometry and unique sampling methods, to assess cellularity in a relevant anatomical compartment- the nasopharynx. Moving forward, over the upcoming influenza seasons, this study design can be used to precisely track immune cells with greater complexity over time and perform functional studies on nasopharyngeal cells. Adaptive immune cell recruitment can be assessed. Extrapolating from studies on NK cells from this patient cohort [52], hyperresponsiveness or functional impairment of nasopharyngeal monocytes and DCs can be investigated. *In situ* differentiation of monocytes into DCs or macrophages can be studied. The virus can be typed using next generation sequencing methods to identify drift variants within a single patient, a single season, and between seasons.

On the clinical aspect of this study, an innate immune profile can be created predicting progression vs resolution of disease. This should include a severity scoring system for seasonal influenza, to help stratify the patients on virological, immunological and clinical findings. By recruiting household contacts of patients [198], or individuals being vaccinated with the LAIV [72, 73, 76, 78], it would be possible to study early immune responses in asymptomatic individuals exposed to IAV, and track them over time. The study setup can be scaled up to multi-centric, longitudinal studies spanning the entire influenza pyramid and season. Studies like these would help prepare logistically for research studies when the next influenza pandemic hits.

In **Paper IV**, we were able to extend the scope of **Paper III**. With further *in vitro* experiments, we can try to address the fate of the different (sorted) monocyte and DC subsets after (likely) exposure to virus, and address their exact contribution to HFRS. Assaying the responses in blood, however, only offers incomplete knowledge

of inflammatory processes as we and others have demonstrated. Performing renal biopsies on patients is only recommended in extremely atypical cases for differential diagnosis. However, urine samples from patients can be analyzed for leukocytes and soluble markers of inflammation. There is a possibility to obtain archived renal biopsy samples from HFRS patients. Using immunohistochemistry and imaging methods, we can study if NCMs adhere to the endothelium in renal vasculature and extravasate into glomerular tissue during hantavirus infection. We can explore for NCM sequestration in the kidneys during HFRS, as seen before in animal models [201, 268]. With Transwell™ systems, we can also assess the individual contribution of different sorted monocyte subsets to the endothelial dysfunction and vascular leakage seen in HFRS.

9 ACKNOWLEDGEMENTS

Over the course of my PhD, I have had the opportunity to work with many wonderful people, who have been involved in my scientific development and supported my personal growth.

First and foremost, I would like to thank my supervisor, **Anna Smed Sörensen**, for every success I have had in the last 5 years. You have trained me as a scientist and been an amazing mentor over these years. You were always patient, kind, encouraging and supportive to me. Being supervised by you has been a thoroughly enjoyable learning experience. Because of you, I can truly say my PhD was a happy one. And I truly appreciated every little note you wrote, and every comment you gave me. You inspire me to be a better scientist and a nicer person, every day. Tack så mycket för allting!

My co-supervisor **Anna Färnert**, thank you so much for being a part of my supervising team, in addition to being an integral part of the flu patient study. You were with Anna and I, every step of the way and made us believe we could do this study. Thank you for also being a great mentor and a role model to me.

My co-supervisors **Johan Grunewald** and **Anders Eklund**, although we did not spend much time together, knowing that I have your support has meant so much to me. Thank you for being a part of my PhD.

And last but not least, my co-supervisor **Clas Ahlm**, thank you for the wonderful discussions and advice about my projects and my future. It has been a true pleasure to know you.

Karin Loré, thank you for being my unofficial co-supervisor over these 5 years. Your regular scientific input and fresh perspective on my studies have been so helpful. Sharing an office with your lab has taught me at least twice as much immunology than I imagined.

Atanu Basu, my mentor for many years, thank you for being a shining beacon of hope and encouragement. Thank you for your guidance, advice and above all, friendship.

Tomas Strandin, you have been a fantastic collaborator for the Finnish hantavirus study. Thank you for being a great colleague and a reliable BSL-3 buddy!

Jonas Klingström, thank you for being a collaborator and teaching me so much about hantaviruses. I have really enjoyed our discussions and working with everyone in your lab.

All the coauthors on my papers, thank you for all your contributions! **Danielle Friberg**, **Magnus Starkhammar** and **Alexander Ahlberg** for collaborating with us and providing us tonsils on demand. **Niclas Johansson** and **Klara Sondén** for training students and tirelessly including patients for the flu patient study. **Jan Albert**, thank you for your keen insights on the flu patient study, and your continued support. **Asghar Muhammad**, thank you for our discussions and helping me with data analysis. **Kimia Maleki**, **Shawon Gupta**, **Johanna Tauriainen**, **Carles Solá-Riera** and **Janne Tynell**, thank you for your help on the hantavirus studies and sharing reagents, expertise and good times. You made early morning experiments at CIM really fun. **Anders Blomberg**, **Gregory Rankin**, **Jamshid Pourazar**, **Magnus Evander Andrea Discacciati**, **Jonas Höijer**, **Matteo Bottai**, **Niklas Björkström**, **Johan Rasmuson**, **Hans-Gustaf Ljunggren**: thank you for being great colleagues on the Swedish hantavirus study. **Olli Vapalahti**, **Antti Vaheri**, **Jukka Mustonen, Sanna Mäki**, and **Rommel Iheozor-Ejiofor**, thank you for a really nice collaboration for the Finnish hantavirus study, and being so warm and

welcoming to me in cold and dark Helsinki. **Jenny Driving** and **Anna Nilsson** (Capio ENT); and **Ingrid** and the many nurses at Karolinska Universitetssjukhuset- thank you for all your help with the patient material. **Jörgen Sälde** and **Tobias Selling**, thank you for helping us expand the flu patient study and fixing the bureaucratic issues we faced.

Nicole Marquardt and **Marlena Scharenberg**, it has been a pleasure to collaborate with you and see an exciting offshoot from the flu patient study.

The PIs at the Division of Immunology and Allergy: thank you for all your scientific input and making it a great working environment. **Marianne van Hage**, **Gunnar Nilsson**, **Susanne Gabrielsson**, **Eduardo Villablanca, Taras Kreslavskiy** and **Joakim Dahlin**, thank you for the great scientific discussions during our Monday seminars. **John Andersson**, thank you for providing welcome relief from tedium, even if it involved terrible movies. Sorry, “cult classics”. **Annelie Tjernlund, Liv Eidsmo**, **Samuel Rhedin**, **Kristina Broliden**, **Andrea Introini**, **Christopher Sundling**, thank you for your comments and discussions over the years.

The **Smed-Sörensen lab**: you have been a joy to work with. **Jens** and **Saskia**, thank you for teaching me all the methods you spent years perfecting. And trusting me to work independently when I was still very new. **Faezzah**, you helped me a lot when I was new to the lab, and you were a great PhD role model. Thank you for the good times! **Sang**, thank you for the fantastic input on the flu and hanta studies. It’s been great working with you. **Rico**, you have been the best PhD colleague/friend imaginable! Not only are you an excellent scientist, but you make everyone around you so happy with your excitement (even at an insane hour in the morning). Thank you for all your comments, suggestions and discussions over the last 5 years. **Meng**, thank you for the wonderful times in the lab, and listening to me talk incessantly. **Sara**, thank you for being an amazing project student and for listening to my pre-coffee talk. I’m so excited for you to take on the flu patient study and take it in exciting new directions. The students I have had the pleasure of working with: **Oliver**, **Adeline**, **Kevin**, **Roosa**, **Patric** and **Björn**: thank you for all your help on my projects. It’s been an incredible learning experience! You have been a pleasure to work with.

Liz and **Frank**, thank you for being great colleagues and always helping me whenever I had a question. **Ang**, you were the first friend I made in Stockholm. Thanks for sharing the KI PhD experience with me! You made everything in the lab more fun. **Christina**, thanks for being my partner in caffeine deprivation, and sharing my excitement over everything nerdy. **Avinash**, thanks for being my friend. But more importantly, thank you for bringing biryani into my life in Stockholm.

The extended lab family/KLASS-T team/**Groggy Frog**: **Klara**, **Mu**, **Isabel**, **Fredrika**, **Gustaf**, **Sebastian**, **Alberto**, **Theresa**, **Annika**, **Josefine**, **Vassilis**, **Lukas**, **Yuanyuan**, **Charlotte**, **Julia**, **Juliane**, **Elena, Philip, Ruben, Mathieu, Tyler**: you made moving to Stockholm, and working in this lab an amazing experience. Thank you for the fikas, the after-works, the fun times and the inside jokes. All the current and past L2:04/U2:J7 colleagues: thank you for a wonderful few years! **Annika Jouper**, thank you for helping me so patiently every time I had an administrative question. All the wonderful people I met at KI, thank you!

Vincent Racaniello, **Dickson Despommier**, **Alan Dove**, **Rich Condit**, **Kathy Spindler** and **Brianne Barker**- thank you for sparking my interest in viruses. And for keeping me company on many a long lab night with weather and virus talk.

My Stockholm family- thank you for being my family. **Emily** and **Omar**- somewhere over the last few years, you have adopted me. I don't have words to express my gratitude for your friendship and love. **Juniper** and **Jasper**, I hope one day you read this and know what it meant to me, to have your warmth be a constant in my life. **Paul**, you are truly my BFF, and I am so grateful you put up with me. I truly don't know if Stockholm would be any fun, without you to explore it with. **Lyndon**, thank you for the adventures, the sarcasm and the sage wisdom. **Mili**, my fellow Asgardian of the galaxy, thanks for geeking out with me over a million little things. **Alex M**, so long and thanks for all the puppies. **Chiara**, **Annie**, **Ruby**, **Nadim**, **Adam**, **Casper**, **Jiji**, **Magnus**, **Alex S**, **Leigh**, **Sara**- thanks for years of fikas, good times and never-ending laughter. **Jon**, Sunday is still Sindhu-day. **Hugo**, tack för att du är min vän. **Ida**, thank you for making my initial days in Stockholm a positive experience.

My Singapore friends- **Sam**, **David**, **Varnica**, **Kurosh**, **Grishma**, **Mana**, **Gourab**, **Kunaal, Ranjit, Lars-** thanks for two incredible years in SG. You made time fly.

My NIV-Pune gang- I'm amazed every day, that over 10 years later, we still remain in touch. You guys motivate me and make me laugh on a daily basis. **Anandi**, thanks for being my friend, sister, peer and family. **Ketaki**, **Tanwee**, **Madhura**, **Shamik, Finny, Prashant**, **Chaitanya**, **Shashi, Shourya, Preeti, Mostafa, Rishank, Mohsen, Minakshi:** thank you for sharing good times, bad times, and always encouraging me to keep on going.

My friends from Mumbai- **Gaya** and **Pravesh**- thank you for all the late night, long chats and for all your insights. **Lulu**, **Suz**, **Sneha**, **Lipika**, **Ishan**, **Prateek**- thanks for picking up right where we leave off. **Riva**, **Sam**, **Nandita, Vivien and Miriam**- thank you for being exceptional teachers, inspiring me to follow my dreams, and for being treasured friends. **Polo**, **Akash, Nick, Sharan, Ganesh**- thanks for years of encouragement when the end seemed so far away. **Apu**, thanks for sharing our mutual obsession and reminding me what youth feels like. **Havi**, **Alo**, **Aarav**, **Jojo**, **Saniya**- you are the very essence of home for me. Thanks for keeping me centered and drowning in hugs. **Joel**, you always believed in me, even on days I couldn't. I definitely wouldn't be here today without you. Thank you for encouraging me go to Sweden and literally packing my bags. Thank you for always showing up for me. **Tejas**, you and I are two sides of the same coin. Thank you for being my confidant, for always listening, and for being my friend forever. **Adi**, thanks for being my champion from the very beginning. Even when we were 3 and all I wanted was to paint walls with water.

Pravali, you are the best little sister anyone could have. Piglet, you make me so proud. You inspire me with your work ethic and the love you have for us. **Mom** and **Dad**, this is not the path you envisioned for me. But you always stood by me, supported me, encouraged me and let me follow my dreams. You let me fly, knowing I always have a home to return to, wherever you are. I am so thankful and privileged to be your daughter. You gave me everything I wanted, including the things I didn't know I needed. Knowing the three of you love and support me unconditionally means the world to me. **Ammamma**, **Aamma**- this one is for you.

And finally, a big thank you to the 151 patients and 63 healthy controls who participated in these studies.

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